Cryptic female choice and gamete-mediated paternal effects in the context of interspecific hybridization

By

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Abstract

The environment in which fertilization takes place can have significant effects on paternity and offspring development. Through cryptic female choice, females can bias paternity to benefit a particular male and "choose" the father of her clutch. These processes might affect offspring development through non-genetic gamete-mediated paternal effects. I chose to examine the impact of cryptic female choice on paternity and offspring development in an externally fertilizing taxa that readily hybridizes, Salmoninae. Hybridization can represent a bad outcome for females with far-reaching effects on offspring phenotype and development. Females can reduce hybridization through conspecific sperm preference, a mechanism of cryptic female choice. What is unknown is the magnitude of conspecific sperm preference and the extent of gamete-mediated parental effects in our study populations and how these effects change in relation to hybridization. Following previous work done with other populations of salmonids, I expected to find evidence for strong conspecific sperm preference and paternal effects. Here, I examined conspecific sperm preference in three species, native brook char (*Salvelinus fontinalis*) and Atlantic salmon (*Salmo salar*), and introduced and invasive brown trout (*Salmo trutta*) and found that while ovarian fluid influenced sperm behavior, this effect did not differ among species. However, while hybridization between Atlantic salmon and brown trout significantly affected offspring development, paternal effects derived from the fertilization environment did not. This implies that females can alter paternity through cryptic female choice without consequences to the offspring. More research is needed in this and other salmonid species and populations to determine if these effects are present across salmonids.

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I would like to thank this opportunity to thank the many people who helped me throughout my master's progress, I would not have been able to do it without your support! I would especially like to thank Craig Purchase for advising, teaching, and giving me the tools I needed to get this work completed and completed well. I would also like to thank my lab mate Madison for all her help getting this done and keeping me sane during long office days. I would like to thank Terry, Coady, Taylor, Sydney, Alex, and the Environment Resources Management Association (ERMA) very much for helping with the field and lab work associated with my project. I would especially like to thank Terry Paul and Darren Ryan of ERMA for collecting and sending gametes from salmon and charr. I would like to acknowledge that a partnership between the Atlantic Salmon Conservation Foundation (ASCF), the Salmonid Association of Eastern Newfoundland (SAEN), and the Environmental Resources Management Association (ERMA) acquired the salmon for use in my experiments. I would like to thank Peter Westley and Travis Van Leeuwen for their service on my graduate committee and for their invaluable advice. I would also like to thank my thesis examiners Ian Jones and Simone Immler for their helpful critiques and comments. I would like to thank my family for supporting me throughout the degree process. I would also like to thank Kelly, Gary, Juliana, and Johanna for being great field partners and making my last Newfoundland summer fun and memorable. I would like to thank Travis and Teagan, Tom Chapman, and Mike for their friendship and for making my time in Newfoundland memorable. I would like to thank Madison, Misha, Emilie, Paul and Joyce, Patrick, Jaclyn, Katherine, Muhammad (otherwise known as the greatest Dungeons and Dragons party of all time), Makayla and so many others for their friendship.

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Appendix Table 3-A1: Block 1 alevin number. Percent hatch was taken from 350 eggs in all treatments except the LAT-SOF-TS treatment which was taken from 186 eggs. Incubation tube density was adjusted at approximately 567 ATU. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

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Appendix Table 3-A3: Block 3 alevin numbers: Percent hatch was taken from 350 eggs. Incubation tube density was adjusted at approximately 567 ATU. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Table 3-A4: Block 4 alevin numbers: Percent hatch was taken from 350 eggs. Incubation tube density was adjusted at approximately 567 ATU. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Table 3-A5: Block 5 alevin numbers: Percent hatch was taken from 350 eggs. Incubation tube density was adjusted at approximately 567 ATU. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Table 3-A6: Block 6 alevin numbers. Percent hatch was taken from 350 eggs. Incubation tube density was adjusted at approximately 567 ATU. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-B1: Logistic regressions of hatch timing for block 1 (solid lines =Salmon x Salmon crosses, dashed =Salmon x Trout crosses, equation=$y = \frac{1}{1+e^{-coefficient)*ATU-(intercept)}}$). Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Sample sizes, intercepts, coefficients, and corresponding Pr(>|z|) values for each treatment were as follows.

SAT-W-SS n=142, -98.084 Pr(>|z|) =<0.001, 0.980 Pr(>|z|) =<0.001

LAT-W-SS n=4, -177.359 Pr(>|z|) =0.006, 1.726 Pr(>|z|) =0.006

LAT-SOF-SS n=173, -96.396 Pr(>|z|) =<0.001, 0.961 Pr(>|z|) =<0.001

LAT-TOF-SS, n=153, -124.191 Pr(>|z|) =<0.001, 1.245 Pr(>|z|) =<0.001

SAT-W-TS n=52, -131.957 Pr(>|z|) =<0.001, 1.432 Pr(>|z|) =<0.001

LAT-W-TS n=8, -182.026 Pr(>|z|) =0.001, 1.963 Pr(>|z|) =0.001

LAT-SOF-TS n=67, -114.735 Pr(>|z|) =<0.001, 1.249 Pr(>|z|) =<0.001

LAT-TOF-TS n=97, -128.257 Pr(>|z|) =<0.001, 1.394 Pr(>|z|) =<0.001

Appendix Figure 3-B2: Logistic regressions of hatch timing for block 2 (solid lines =Salmon x Salmon crosses, dashed =Salmon x Trout crosses, equation=$y = \frac{1}{1+e^{-coefficient)*ATU-(intercept)}}$). Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Sample sizes, intercepts, coefficients, and corresponding Pr(>|z|) values for each treatment were as follows.

SAT-W-SS n=134, -144.055 Pr(>|z|) =<0.001, 1.424 Pr(>|z|) =<0.001

LAT-W-SS n=127, -200.134 Pr(>|z|) =<0.001, 1.972 Pr(>|z|) =<0.001

LAT-SOF-SS n=131, -152.024 Pr(>|z|) =<0.001, 1.498 Pr(>|z|) =<0.001

LAT-TOF-SS, n=163, -109.779 Pr(>|z|) =<0.001, 1.086 Pr(>|z|) =<0.001

SAT-W-TS n=36, -148.519 Pr(>|z|) =<0.001, 1.582 Pr(>|z|) =<0.001

LAT-W-TS n=16, -37.478 Pr(>|z|) =<0.001, 0.393 Pr(>|z|) =<0.001

LAT-SOF-TS n=13, =109.435 Pr(>|z|) =0.001, 1.159 Pr(>|z|) =0.001

LAT-TOF-TS n=107, -178.184 Pr(>|z|) =<0.001, 1.905 Pr(>|z|) =<0.001

Appendix Figure 3-B3: Logistic regressions of hatch timing for block 3 (solid lines =Salmon x Salmon crosses, dashed =Salmon x Trout crosses, equation=$y = \frac{1}{1+e^{-coefficient)*ATU-(intercept)}}$). Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Sample sizes, intercepts, coefficients, and corresponding Pr(>|z|) values for each treatment were as follows.

SAT-W-SS n=84, -90.930 Pr(>|z|) =<0.001, 0.833 Pr(>|z|) =<0.001

LAT-W-SS n=0

LAT-SOF-SS n=135, -103.213 Pr(>|z|) =<0.001, 0.940 Pr(>|z|) =<0.001

LAT-TOF-SS, n=0

SAT-W-TS n=117, -178.023 Pr(>|z|) =<0.001, 1.781 Pr(>|z|) =<0.001

LAT-W-TS n=12, -140.515 Pr(>|z|) =0.001, 1.382 Pr(>|z|) =0.001

LAT-SOF-TS n=64, -162.992 Pr(>|z|) =<0.001, 1.626 Pr(>|z|) =<0.001

LAT-TOF-TS n=112, -207.028 Pr(>|z|) =<0.001, 2.074 Pr(>|z|) =<0.001

Appendix Figure 3-B4: Logistic regressions of hatch timing for block 4 (solid lines =Salmon x Salmon crosses, dashed =Salmon x Trout crosses, equation=$y = \frac{1}{1+e^{-coefficient)*ATU-(intercept)}}$). Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Sample sizes, intercepts, coefficients, and corresponding Pr(>|z|) values for each treatment were as follows.

SAT-W-SS n=125, -134.571 Pr(>|z|) =<0.001, 1.246 Pr(>|z|) =<0.001

LAT-W-SS n=68, -158.789 Pr(>|z|) =<0.001, 1.467 Pr(>|z|) =<0.001

LAT-SOF-SS n=127, -125.559 Pr(>|z|) =<0.001, 1.161 Pr(>|z|) =<0.001

LAT-TOF-SS, n=112, -65.881 Pr(>|z|) =<0.001, 0.608 Pr(>|z|) =<0.001

SAT-W-TS n=32, -137.860 Pr(>|z|) =<0.001, 1.371 Pr(>|z|) =<0.001

LAT-W-TS n=1, -3105.240 Pr(>|z|) =0.988, 31.21 Pr(>|z|) =0.988

LAT-SOF-TS n=71, -216.635 Pr(>|z|) =<0.001, 2.151 Pr(>|z|) =<0.001

LAT-TOF-TS n=10, -136.988 Pr(>|z|) =0.001, 1.369 Pr(>|z|) =0.001

Appendix Figure 3-B5: Logistic regressions of hatch timing for block 5 (solid lines =Salmon x Salmon crosses, dashed =Salmon x Trout crosses, equation=$y = \frac{1}{1+e^{-coefficient)*ATU-(intercept)}}$). Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Sample sizes, intercepts, coefficients, and corresponding Pr(>|z|) values for each treatment were as follows.

SAT-W-SS n=133, -102.031 Pr(>|z|) =<0.001, 1.021 Pr(>|z|) =<0.001

LAT-W-SS n=22, -123.830 Pr(>|z|) =<0.001, 1.218 Pr(>|z|) =<0.001

LAT-SOF-SS n=154, -143.598 Pr(>|z|) =<0.001, 1.436 Pr(>|z|) =<0.001

LAT-TOF-SS, n=160, -141.0346 Pr(>|z|) =<0.001, 1.411 Pr(>|z|) =<0.001

SAT-W-TS n=105, -154.025 Pr(>|z|) =<0.001, 1.683 Pr(>|z|) =<0.001

LAT-W-TS n=6, -114.598 Pr(>|z|) =<0.001, 1.232 Pr(>|z|) =<0.001

LAT-SOF-TS n=1, -2980.69 Pr(>|z|) =0.989, 31.54 Pr(>|z|) =0.989

LAT-TOF-TS n=13, -213.452 Pr(>|z|) =<0.001, 2.317 Pr(>|z|) =<0.001

Appendix Figure 3-B6: Logistic regressions of hatch timing for block 6 (solid lines =Salmon x Salmon crosses, dashed =Salmon x Trout crosses, equation=$y = \frac{1}{1+e^{-coefficient)*ATU-(intercept)}}$). Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Sample sizes, intercepts, coefficients, and corresponding Pr(>|z|) values for each treatment were as follows.

SAT-W-SS n=169, -87.672 Pr(>|z|) =<0.001, 0.901 Pr(>|z|) =<0.001

LAT-W-SS n=170, -121.177 Pr(>|z|) =<0.001, 1.225 Pr(>|z|) =<0.001

LAT-SOF-SS n=143, -122.611 Pr(>|z|) =<0.001, 1.257 Pr(>|z|) =<0.001

LAT-TOF-SS, n=160, -90.043 Pr(>|z|) =<0.001, 0.917 Pr(>|z|) =<0.001

SAT-W-TS n=100, -116.555 Pr(>|z|) =<0.001, 1.281 Pr(>|z|) =<0.001

LAT-W-TS n=1, -2924.93 Pr(>|z|) =0.989, 31.62 Pr(>|z|) =0.989

LAT-SOF-TS n=149, -187.044 Pr(>|z|) =<0.001, 2.067 Pr(>|z|) =<0.001

LAT-TOF-TS n=125, -174.468 Pr(>|z|) =<0.001, 1.928 Pr(>|z|) =<0.001

Appendix Figure 3-C1: Standard lengths of fish from block 1 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=73, LAT-W-SS n=3, LAT-SOF-SS n=88, LAT-TOF-SS n=78, SAT-W-TS n=26, LAT-W-TS n=8, LAT-SOF-TS n=44, LAT-TOF-TS n=52. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-C2: Standard lengths of fish from block 2 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as

follows; SAT-W-SS n=87, LAT-W-SS n=68, LAT-SOF-SS n=69, LAT-TOF-SS n=81, SAT-W-TS n=36, LAT-W-TS n=13 LAT-SOF-TS n=13, LAT-TOF-TS n=58. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-C3: Standard lengths of fish from block 3 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=24, LAT-W-SS n=0, LAT-SOF-SS n=68, LAT-TOF-SS n=0, SAT-W-TS n=59, LAT-W-TS n=12, LAT-SOF-TS n=31, LAT-TOF-TS n=69. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-C4: Standard lengths of fish from block 4 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=62, LAT-W-SS n=36, LAT-SOF-SS n=52, LAT-TOF-SS n=51, SAT-W-TS n=16, LAT-W-TS n=0 LAT-SOF-TS n=47, LAT-TOF-TS n=7. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-C5: Standard lengths of fish from block 5 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=65, LAT-W-SS n=22, LAT-SOF-SS n=79, LAT-TOF-SS n=87, SAT-W-TS n=62, LAT-W-TS n=7 LAT-SOF-TS n=1, LAT-TOF-TS n=13. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-C6: Standard lengths of fish from block 6 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data Sample sizes for each treatment were as follows; SAT-W-SS n=90, LAT-W-SS n=47, LAT-SOF-SS n=82, LAT-TOF-SS n=84, SAT-W-TS n=45, LAT-W-TS n=1 LAT-SOF-TS n=66, LAT-TOF-TS n=55. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-D1: The head lengths of fish from block 1 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=73, LAT-W-SS n=3, LAT-SOF-SS n=88, LAT-TOF-SS n=79, SAT-W-TS n=25, LAT-W-TS n=8, LAT-SOF-TS n=44, LAT-TOF-TS n=52. Legend: SAT=Short Activation (0 seconds),

LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-D2: Head lengths of fish from block 2 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=87, LAT-W-SS n=68, LAT-SOF-SS n=72, LAT-TOF-SS n=80, SAT-W-TS n=36, LAT-W-TS n=14, LAT-SOF-TS n=13, LAT-TOF-TS n=59. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-D3: Head lengths of fish from block 3 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=26, LAT-W-SS n=0, LAT-SOF-SS n=68, LAT-TOF-SS n=0, SAT-W-TS n=60, LAT-W-TS n=12, LAT-SOF-TS n=32, LAT-TOF-TS n=69. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-D4: Head lengths of fish from block 4 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=62, LAT-W-SS n=37, LAT-SOF-SS n=52, LAT-TOF-SS n=53, SAT-W-TS n=17, LAT-W-TS n=0, LAT-SOF-TS n=46, LAT-TOF-TS n=7. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-D5: Head lengths of fish from block 5 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=62, LAT-W-SS n=20, LAT-SOF-SS n=78, LAT-TOF-SS n=84, SAT-W-TS n=51, LAT-W-TS n=7, LAT-SOF-TS n=1, LAT-TOF-TS n=13. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-D6: Head lengths of fish from block 6 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=90, LAT-W-SS n=47, LAT-SOF-SS n=82, LAT-TOF-SS n=85, SAT-W-TS n=46, LAT-W-TS n=1, LAT-SOF-TS n=66, LAT-TOF-TS n=55. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-E1: Caudal fin ray counts of fish from block 1 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=71, LAT-W-SS n=3, LAT-SOF-SS n=85, LAT-TOF-SS n=77, SAT-W-TS n=25, LAT-W-TS n=6, LAT-SOF-TS n=44, LAT-TOF-TS n=49. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-E2: Caudal fin ray counts of fish from block 2 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=87, LAT-W-SS n=64, LAT-SOF-SS n=70, LAT-TOF-SS n=78, SAT-W-TS n=36, LAT-W-TS n=14, LAT-SOF-TS n=13, LAT-TOF-TS n=56. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-E3: Caudal fin ray counts of fish from block 3 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=24, LAT-W-SS n=0, LAT-SOF-SS n=68, LAT-TOF-SS n=0, SAT-W-TS n=58, LAT-W-TS n=12, LAT-SOF-TS n=32, LAT-TOF-TS n=71. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-E4: Caudal fin ray counts of fish from block 4 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=61, LAT-W-SS n=34, LAT-SOF-SS n=51, LAT-TOF-SS n=50, SAT-W-TS n=16, LAT-W-TS n=0, LAT-SOF-TS n=43, LAT-TOF-TS n=7. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-E5: Caudal fin ray counts of fish from block 5 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=62, LAT-W-SS n=20, LAT-SOF-SS n=78, LAT-TOF-SS n=84, SAT-W-TS n=51, LAT-W-TS n=7, LAT-SOF-TS n=1, LAT-TOF-TS n=13. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-E6: Caudal fin ray counts of fish from block 6 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as

follows; SAT-W-SS n=88, LAT-W-SS n=45, LAT-SOF-SS n=82, LAT-TOF-SS n=83, SAT-W-TS n=41, LAT-W-TS n=14 LAT-SOF-TS n=63, LAT-TOF-TS n=54. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-F1: Standard lengths of fish from block 1 at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=133, LAT-W-SS n=0, LAT-SOF-SS n=171, LAT-TOF-SS n=130, SAT-W-TS n=37, LAT-W-TS n=0 LAT-SOF-TS n=36, LAT-TOF-TS n=104. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-F2: Standard lengths of fish from block 2 at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=118, LAT-W-SS n=144, LAT-SOF-SS n=134, LAT-TOF-SS n=115, SAT-W-TS n=0, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=115. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-F3: Standard lengths of fish from block 3 at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=49, LAT-W-SS n=0, LAT-SOF-SS n=49, LAT-TOF-SS n=0, SAT-W-TS n=49, LAT-W-TS n=0 LAT-SOF-TS n=49, LAT-TOF-TS n=50. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-F4: Standard lengths of fish from block 4 at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=50, LAT-SOF-SS n=50, LAT-TOF-SS n=50, SAT-W-TS n=37, LAT-W-TS n=0 LAT-SOF-TS n=50, LAT-TOF-TS n=0. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-F5: Standard lengths of fish from block 5 at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=0, LAT-SOF-SS n=50, LAT-TOF-SS n=50, SAT-W-TS n=50, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=0. Legend: SAT=Short Activation (0 seconds),

LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-F6: Standard lengths of fish from block 6 at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=50, LAT-SOF-SS n=50, LAT-TOF-SS n=50, SAT-W-TS n=50, LAT-W-TS n=0 LAT-SOF-TS n=50, LAT-TOF-TS n=49. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-G1: Proportion of block 1 fish with separated adipose fins at 800 ATU. Triangles represent the means for each treatment. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=132, LAT-W-SS n=0, LAT-SOF-SS n=155, LAT-TOF-SS n=105, SAT-W-TS n=41, LAT-W-TS n=0 LAT-SOF-TS n=32, LAT-TOF-TS n=105. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-G2: Proportion of block 2 fish with separated adipose fins at 800 ATU. Triangles represent the means for each treatment. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=80, LAT-W-SS n=128, LAT-SOF-SS n=113, LAT-TOF-SS n=166, SAT-W-TS n=0, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=79. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-G3: Proportion of block 3 fish with separated adipose fins at 800 ATU. Triangles represent the means for each treatment. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=49, LAT-W-SS n=0, LAT-SOF-SS n=47, LAT-TOF-SS n=0, SAT-W-TS n=47, LAT-W-TS n=0 LAT-SOF-TS n=47, LAT-TOF-TS n=49. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-G4: Proportion of block 4 fish with separated adipose fins at 800 ATU. Triangles represent the means for each treatment. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=48, LAT-W-SS n=48, LAT-SOF-SS n=48, LAT-TOF-SS n=48, SAT-W-TS n=37, LAT-W-TS n=0 LAT-SOF-TS n=41, LAT-TOF-TS n=0. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-G5: Proportion of block 5 fish with separated adipose fins at 800 ATU. Triangles represent the means for each treatment. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=48, LAT-W-SS n=0, LAT-SOF-SS n=40, LAT-TOF-SS n=49, SAT-W-TS n=49, LAT-W-TS n=0 LAT-SOF-TS

n=0, LAT-TOF-TS n=0. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-G6: Proportion of block 6 fish with separated adipose fins at 800 ATU. Triangles represent the means for each treatment. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50 LAT-W-SS n=49, LAT-SOF-SS n=48, LAT-TOF-SS n=49, SAT-W-TS n=50, LAT-W-TS n=0 LAT-SOF-TS n=47, LAT-TOF-TS n=46. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-H1: Parr mark counts from block 1 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=104, LAT-W-SS n=0, LAT-SOF-SS n=174, LAT-TOF-SS n=136, SAT-W-TS n=4, LAT-W-TS n=0 LAT-SOF-TS n=35, LAT-TOF-TS n=77. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-H2: Parr mark counts from block 2 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=117, LAT-W-SS n=144, LAT-SOF-SS n=135, LAT-TOF-SS n=179, SAT-W-TS n=0, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=116. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-H3: Parr mark counts from block 3 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=0, LAT-SOF-SS n=50, LAT-TOF-SS n=0, SAT-W-TS n=50, LAT-W-TS n=0 LAT-SOF-TS n=50, LAT-TOF-TS n=50. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-H4: Parr mark counts from block 4 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=50, LAT-SOF-SS n=50, LAT-TOF-SS n=50, SAT-W-TS n=50, LAT-W-TS n=0 LAT-SOF-TS n=50, LAT-TOF-TS n=0. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-H5: Parr mark counts from block 5 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin

shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=0, LAT-SOF-SS n=50, LAT-TOF-SS n=50, SAT-W-TS n=50, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=0. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-H6: Parr mark counts from block 6 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=50, LAT-SOF-SS n=50, LAT-TOF-SS n=50, SAT-W-TS n=50, LAT-W-TS n=0 LAT-SOF-TS n=50, LAT-TOF-TS n=50. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-I1: Caudal ray counts from block 1 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=146, LAT-W-SS n=0, LAT-SOF-SS n=174, LAT-TOF-SS n=131, SAT-W-TS n=45, LAT-W-TS n=0 LAT-SOF-TS n=36, LAT-TOF-TS n=107. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-I2: Caudal ray counts from block 2 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=91, LAT-W-SS n=140, LAT-SOF-SS n=134, LAT-TOF-SS n=174, SAT-W-TS n=0, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=83. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-I3: Caudal ray counts from block 3 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=49, LAT-W-SS n=0, LAT-SOF-SS n=47, LAT-TOF-SS n=0, SAT-W-TS n=49, LAT-W-TS n=0 LAT-SOF-TS n=50, LAT-TOF-TS n=49. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-I4: Caudal ray counts from block 4 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=49, LAT-W-SS n=49, LAT-SOF-SS n=50, LAT-TOF-SS n=49, SAT-W-TS n=35, LAT-W-TS n=0 LAT-SOF-TS n=48, LAT-TOF-TS n=0. Legend: SAT=Short Activation (0 seconds),

LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-I5: Caudal ray counts from block 5 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=0, LAT-SOF-SS n=50, LAT-TOF-SS n=50, SAT-W-TS n=49, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=0. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Appendix Figure 3-I6: Caudal ray counts from block 6 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=48, LAT-SOF-SS n=48, LAT-TOF-SS n=49, SAT-W-TS n=49, LAT-W-TS n=0 LAT-SOF-TS n=47, LAT-TOF-TS n=47. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Chapter 1. Introduction

Contextual background

Hybridization

Hybridization describes successful reproduction and offspring development resulting from a mating between different species (Schwenk et al., 2008). Successful hybridization can lead to speciation (Abbott et al., 2013) or genetic introgression of one species' genes into another species (Harrison & Larson, 2014), but this is not the norm. It is more common for hybrid matings to result in lowered reproductive success compared to pure-species parental crosses because offspring are often sterile or not produced at all due to factors such as errors in gene expression or differences in chromosome number (Maheshwari & Barbash, 2011; Ortíz-Barrientos et al., 2007). In these situations, hybrid matings represent wasted reproductive

energy for both sexes (Bateman, 1948; Trivers, 1972), whether surviving offspring are produced or not. Although both sexes are affected by this waste, females incur the greater energetic cost for each hybrid mating (Emery Thompson & Georgiev, 2014) for physical and behavioral reasons. While males produce more gametes than females, and can require large amounts of energy to create and maintain (Evans et al., 2003); individual eggs are exponentially larger than sperm, which means that the female must invest more energy per gamete than a male (Hayward & Gillooly, 2011). Consequently, female mating opportunities are limited by the number of eggs she can produce, while males are limited by the number of females they can mate with (Bateman, 1948). Therefore, males have little to lose for a single mating and thus might seek heterospecific females, while females should avoid heterospecific matings and fertilizations.

Since energetic investment into gamete production limits females more than males, males should maximize their opportunities to mate, while females should be more selective than males when selecting a mate to mitigate the risk of a wasted opportunity (Shuster, 2009). This can be exaggerated in taxa with parental care, where the female, with few exceptions (Muldal et al., 1986), is also responsible for caring for the offspring (Emery Thompson & Georgiev, 2014; Froy et al., 2016; Goymann et al., 2016). These choices are described as processes of sexual selection (Kokko & Jennions, 2008; Pianka, 1970).

Sexual selection

Sexual selection occurs via differential reproductive success due to competition between members of the same sex or mate choice for the opposite sex (Andersson, 1994;

Darwin, 1871; Jones & Ratterman, 2009; Kuijper et al., 2012). Before mating, females select a male based on desired phenotypes (Clutton-Brock & McAuliffe, 2009), which can lead to direct benefits such as protection (Galimberti et al., 2000), parental care (Préault et al., 2005), and/or nuptial gifts (Albo et al., 2014). Mate choice can also have indirect benefits, where desired characteristics act as honest signals of male fitness, which can positively benefit offspring (Castillo & Arce, 2020; Jones & Ratterman, 2009). These benefits to offspring can include increased survival (Fisher, 1915; Gowaty et al., 2010), size (Cothran, 2008), or desirability (Head et al., 2005; Klemme et al., 2008) to females later in life. In most cases, females chose a conspecific male (Ryan, 1980), but in some situations, females mate with heterospecific males whether she chooses them or not (Willis, 2013).

Females may mate with another species due to male availability (Grant & Grant, 1997), preference for unfamiliar male traits (Parker & Partridge, 1998), or pre-existing preference for traits present in heterospecific males (Ryan, 1998). An example of female mate choice leading to hybridization is found in swordtail fish (*Xiphophorus*), where females select for males with larger tail ornaments, regardless of the species of the male (Basolo, 1990). Hybrid matings can also occur when the female does not want them to. When females decline to mate with a heterospecific male, males can circumvent female mate choice by using alternative reproductive tactics such as sneaking (Esteve, 2005; Gross, 1996) or female mimicry (Mason & Crews, 1985; Norman et al., 1999). These alternative reproductive tactics have been shown to contribute to interspecific hybridization in a variety of taxa including, damselflies (Nomakuchi & Higashi, 1996), toads (Gergus et al., 1999), and fishes (Garcia-Vazquez et al., 2002; Neat, 2001). Despite these efforts made by males to steal fertilizations, females have mechanisms to bias

paternity after mating, but before fertilization occurs, to favor a particular male in sperm competition (Birkhead & Pizzari, 2002).

As implied above, these mechanisms are only present in situations where there is more than one potential father, including in polyandry, where a female mates with multiple males to increase her chances of mating with a high-quality male (Garcia-Gonzalez & Simmons, 2005). Male post-ejaculatory sexual selection, or sperm competition, occurs when two or more ejaculates attempt to fertilize a clutch of eggs and involves male modulation of sperm quality, ejaculate size, copulation, or other physical and/or chemical means of hindering other males' chances of fertilization (Edward et al., 2015; Parker, 1970). Females can bias the outcome of sperm competition through cryptic female choice (Firman et al., 2017). In situations of hybridization, this bias favors conspecific sperm, known in the literature as conspecific sperm preference (Howard et al., 2009). In internally fertilizing taxa, these manipulations include choosing the order and origin of subsequent inseminations (Firman et al., 2017; Xu & Wang, 2010), ejecting ejaculates of unwanted males (Pizzari & Birkhead, 2000), differentially storing ejaculates in more or less favorable sites (Schnakenberg et al., 2012), and mechanically contracting the reproductive tract to facilitate sperm movement (Friesen et al., 2016; Troisi & Carosi, 1998). Internal and external fertilizers can affect paternity by chemically altering the environment to help or hinder sperm (Holman & Snook, 2008; Robertson, 2007).

In external fertilizers, who solely rely on chemical alterations of the environment to affect paternity after mating, mechanisms of cryptic female choice and conspecific sperm preference outside of not releasing gametes around the "wrong" male are limited to chemical components released with the eggs (Gasparini et al., 2020). Cryptic female choice in these taxa

is either intrinsically linked to the egg (ex. chemotaxis in response to egg proteins in sea urchins, Chang et al., 2013) or chemical compounds released with the egg in the form of egg water in aquatic invertebrates (Lymbery et al., 2017) or ovarian fluid in fishes (Gasparini & Pilastro, 2011). These compounds have been linked to changes in sperm swimming behaviour that then bias the winner of sperm competition (Alonzo et al., 2016; Poli et al., 2019; Yeates et al., 2013). These female-mediated changes are particularly important in broadcast spawning taxa, where multiple species may release gametes indiscriminately at the same time, such as in mussels (*Mytilus*) or when external fertilizers release gametes "at" one another, as is the case in some fishes. In these situations, the only opportunity to bias paternity once gametes are released stems from this mechanism (Klibansky & McCartney, 2014).

This form of cryptic female choice is thought to be the ancestral form of sexual selection when broadcast spawning was the typical form of reproduction (Marshall & Bolton, 2007; Parker, 2014). Darwinian sexual selection (Darwin, 1871), where females choose showy males as mates, is the more recent derived form of sexual selection (Parker, 2014). Cryptic female choice is stronger in organisms with the more robust pre- and post-mating choice present in Darwinian selection and internal fertilization, respectively, because the female has more control over who mates with her. However, there are still robust modifications to the environment of fertilization and paternity in this ancestral form of sexual selection. Evidence of this strength has been found in low rates of hybridization in assemblages of closely related aquatic invertebrates (Klibansky & McCartney, 2014) and examinations of sperm reaction to cryptic female cues (Yeates et al., 2013). These changes to the fertilization environment provide

opportunities for parental effects to arise, which alter offspring development and/or phenotype.

Parental effects

Mothers and fathers have differing effects on their offspring's development in addition to the genes they contribute. These parental effects can be direct through processes such as resource provisioning, parental care, and natal environment (Qvarnström & Price, 2001), or indirect through epigenetic contributions from the parent (Kappeler & Meaney, 2010). In most taxa, the offspring's phenotype is influenced more strongly by the mother's phenotype and environment, particularly in early life history stages, than the father's (Heath et al., 1999; Mousseau & Fox, 1998). This disparity in the visibility of maternal effects over paternal effects can be linked to anisogamy. Since the egg is much larger than the sperm, it contributes more energy to the development, size, and early growth of the offspring (Bernardo, 1996). As offspring mature maternal effects on development are minimized, because offspring become more independent with age (Mousseau & Fox, 1998). Paternal effects are present in early life history; however, it is challenging to isolate paternal effects in early development because of female processes such as cryptic female choice and female energy provisioning (Mousseau & Fox, 1998).

Processes like cryptic female choice alter the environment and, therefore, the experiences of the sperm before fertilization. For example, modulation of sperm behaviour provides an opportunity for gamete-mediated paternal effects to alter offspring development or phenotype (Evans et al., 2019). Paternal effects can result from epigenetic modifications

such as DNA methylation, the addition of non-coding sperm RNAs, and/or histone modification, or changes to the ejaculate that stem from modification over time (Evans et al., 2019). Paternal contribution to the egg is not limited to genetic material, but includes non-genetic sperm factors such as proteins, RNA's, and chromatin modifications (Immler, 2018), and the donation of the centriole, which is a critical component of a viable embryo (Schatten & Sun, 2010). Despite this comparatively small contribution, factors that do not alter the genetic code such as the conditions sperm are exposed to before fertilization (Gasparini et al., 2018; Ritchie & Marshall, 2013), or the male's quality (Hosken et al., 2003) can drastically affect the rate of offspring development. This can be due to epigenetics (Donkin & Barrès, 2018), haploid selection - or selection for a given sperm in an ejaculate (Immler et al., 2014). Paternal quality has also been linked to increased survival (Gowaty et al., 2010), body size (Cothran, 2008), and reproductive production of offspring in later life (Klemme et al., 2008).

Main theme of thesis and key research questions

In the context of hybridization, it is unknown the effect, if any, that mechanisms enabling conspecific sperm preference have on gamete-mediated paternal effects. It is also unknown if the strengths of these conspecific sperm preferences vary between taxa in situations with more than one species of potential heterospecific father. While there can be a preference between two species (Yeates et al., 2013), any variations in the strength of that preference depending on factors such as taxonomic relatedness or likelihood of reproductive interaction across more than two species is unknown. Adding a new species into a reproductive system can drastically change the species' reproductive dynamics within that system because introductions of new species to an environment can result in interbreeding (Hubbs, 1955) and,

therefore, more opportunities for sperm to be exposed to conspecific sperm preference. It is also unknown if the effects of this conspecific sperm preference or female alteration of the fertilization environment leads to gamete-mediated paternal effects on offspring. In this thesis, I seek to examine the intersection of cryptic female choice and gamete-mediated paternal effects under the context of interspecific hybridization. I investigate this in a study taxon, Salmoninae, that readily hybridizes and has documented evidence of both gamete-mediated paternal effects and conspecific sperm preference.

Study System

Why Salmoninae

Salmonids are a group of diverse teleost fishes with complex life histories and reproductive behaviours. These fishes exhibit mutual mate choice, where males court the females and females then choose to release eggs in proximity to that chosen male (Auld et al., 2019). However, a wide variety of reproductive tactics, including sneaker males, which sidestep this female choice and may lead to increased rates of hybridization (Garcia-Vazquez et al., 2002). In salmonids, sneaker males represent a smaller male phenotype that steals fertilizations from larger dominant males. Even though sneaker males have less semen than dominant males, the semen they do have is of higher quality (Vladić et al., 2010; Young et al., 2013), giving the sneaker phenotype a chance to fertilize more eggs than would be expected by volume in salmonids (Young et al., 2013). Cryptic female choice is thought to act as a bias against this (Young et al., 2013). This becomes important because of the high frequency of interspecific hybridization within this taxon (Hendry & Stearns, 2003). Salmonids have also been

documented to have environmentally mediated paternal effects (Immler et al., 2014) that I suspect could be altered by mechanisms of cryptic female choice.

Parental effects in salmonids

Parental effects on offspring in salmonids have been documented in several species, including those in the genera *Oncorhynchus*, *Salmo*, and *Salvelinus* (Kamler, 2005). Maternal effects such as the linkage of offspring size to egg size are common throughout the salmonids (Einum & Fleming, 1999; Heath et al., 1999; Kristjánsson & Vøllestad, 1996; Penney et al., 2018). The offspring's initial size and growth are limited by the size of the egg as well as the amount and quality of nutrients within the yolk (Einum & Fleming, 1999). In brown trout (*Salmo trutta),* the size of the eggs has been strongly linked to offspring size at hatch, the rate of development, metabolic rate after emergence, and survival after hatch (Bagenal, 1969; Einum & Fleming, 1999; Ojanguren et al., 1996; Régnier et al., 2010). Atlantic salmon (*Salmo salar*) and brown trout also exhibit strong maternal effects because the female chooses the physical and thermal environment (e.g., a groundwater seep in a stream vs. an area with no groundwater influence) in which her eggs will develop. This means that the environmental factors that act on the eggs are somewhat controlled by maternal decisions before fertilization (Heggberget et al., 1988). Paternal effects are present in the salmonids but are less obvious.

This means paternal effects are much more difficult to identify in early life history, but they may play a crucial role in the development of offspring. Paternal effects are typically thought of as adult experiences that change offspring. Examples of these types of paternal effects have been found in brown trout and Arctic char (*Salvelinus alpinus*), where paternal

coloration was found to influence the offspring's viability (Wedekind et al., 2008) and growth rate after hatch (Eilertsen et al., 2008), respectively. Paternal effects are not limited to adult experiences; the experiences of the gametes can also change offspring development. Environmental factors such as the time it takes for the sperm to fertilize the egg have been noted to influence development (Immler et al., 2014). Both types of paternal effects can be altered by mate choice (selection for male coloring) and the aforementioned female modulation of the environment. In this thesis, I examine these effects in the context of interspecific hybridization.

It is unknown is how these parental effects change in the context of hybridization among species. Hybridization between individuals of different species changes the genome and hugely affects offspring phenotype and development, arguably much more than paternal effects. Salmonids also have robust defenses against hybridization through conspecific sperm preference to avoid the consequences of hybrid fertilization.

Hybridization in salmonids

Salmonid intergeneric and interspecific hybridization has been documented in a wide variety of systems across the world (Chevassus, 1979; Dangel et al., 1973). Work has been done examining the viability of various crosses between species of salmonids (Chevassus, 1979; Ito et al., 2006), but results vary based on the location of the populations tested. This is due to chromosomal differences between populations that directly affect the viability of offspring (Hartley, 1987). The relative ease of hybridization between species is also compounded by the status of salmonids as invasive species.

Novel hybridization is one of many ecological impacts of invasion and can directly threaten the viability of populations (Krueger & May, 1991; Poulos, 2019). Novel invasions of salmonids have been tied to increased rates of hybridization. This leads to both ecological impacts if hybrids survive to adulthood (ex. competition for food resources [Cucherousset et al., 2007]) and energetic waste resulting from fertilizations stolen from viable conspecific males (Hochkirch et al., 2007). In both cases, hybridization can lead to the imperiling or extirpation of native populations if sufficiently widespread. The study system used in this thesis includes potential heterogeneric, and congeneric heterospecific hybridization in the context of recent species invasion.

I chose three species of Newfoundland salmonids, native brook char (*Salvelinus fontinalis*) and Atlantic salmon, and introduced and invasive brown trout; that can potentially hybridize with varying degrees of likelihood and success (Table 1-1). The likelihood of reproductive interactions occurring depends on the length of overlap between spawning periods and the timing of reproduction. Because the effectiveness of cryptic female choice to prevent hybridization may be variable, hybridization among brown trout, Atlantic salmon, and brook char may occur given that all three species occur in similar habitats and spawn at similar times (Table 1-1). These fish have interacted to varying degrees throughout their evolutionary histories.

Atlantic salmon are native to both North America and Europe, while brook char are only native to North America and brown trout are only native to Europe. North American Atlantic salmon and brook char have been separated evolutionarily from brown trout for 600,000 years (Lenhert et al., 2020). However, the introduction and invasion of brown trout into North

America has brought these populations into contact (MacCrimmon and Marshall, 1968). The populations used in this work exhibit various degrees of separation from each other. The Brook char used in this study come from an isolated lake in the Exploits River watershed (Gibson et al., 1999) and have not been exposed to either Atlantic salmon or brown trout. While our Atlantic salmon occur in the same watershed as the brook char system, it is unknown the degree of contact they may have. Brown trout and brook char have been in contact in Windsor Lake since brown trout were introduced in 1883 (Hustins, 2007).

While brook char have been underrepresented in this type of research, Atlantic salmon and brown trout have been studied in the context of conspecific sperm preference (Yeates et al., 2013).

Hybridization in these study species

Ovarian fluid has been linked to conspecific sperm preference in European populations of Atlantic salmon and brown trout (Yeates et al., 2013). In these taxa, sperm activated in a conspecific ovarian fluid swim longer and with greater velocity compared to sperm activated in a heterospecific ovarian fluid. Curiously, this has been shown to have nothing to do with the species of the egg. In fact, when comparing two species of sperm in sperm competition, if an ovarian fluid of a different species is used to coat eggs, then heterospecific sperm to the eggs get the velocity and longevity advantage over conspecific sperm and achieve a greater number of fertilizations (Yeates et al., 2013). In naturally co-occurring Atlantic salmon and brown trout in Europe, these congeneric fish have a long ecological/evolutionary history together and are often found in the same rivers, utilize similar spawning habitats, and spawn at similar times

(Garcia-Vazquez et al., 2004). In Newfoundland, where Atlantic salmon are native and brown trout are introduced and invasive, the frequency of juvenile hybrids is approximately five percent and is like those in naturally sympatric populations in Europe, where hybrid frequency ranges from 2 to 13 percent (McGowan and Davidson, 1992b). While hybrid occurrence appears to be low, survival is approximately 80-90% (McGowan & Davidson, 1992a; Poulos, 2019). The above is an example of invasion leading to novel hybridization.

Brown trout were introduced to Newfoundland around St. John's at the end of the 19th century (Hustins, 2007). Since then, sea-run brown trout have invaded watersheds throughout the Avalon Peninsula (Westley & Fleming, 2011). This process has brought previously isolated populations of brook char and Atlantic salmon in contact with brown trout, which due to habitat and behavioral overlap (Cunjak & Power, 1986; Fausch & White, 1981; Jansson et al., 1991; McGowan & Davidson, 1992b) can result in reproductive interactions and potentially hybridization (Cucherousset et al., 2007; Jansson et al., 1991; Sorensen et al., 1995; Verspoor, 1988). Brown trout also produce heterogeneric hybrids with brook char, known as tiger trout (Buss and Wright, 1958). Natural hybridization between native Atlantic salmon and brook char, which is also a heterogeneric cross, has not been reported.

Atlantic salmon and brook char are native to Newfoundland (Page et al., 1991) and overlap in spawning time and habitat; however, no documented hybrids in the wild have been reported. Hybrids of the two in laboratory settings suggest that hybridization is possible, but offspring exhibit poor survival (Chevassus, 1979). Therefore, the absence of hybrids in the wild could be because of poor survival, fertilization isolation through conspecific sperm preference, or pre-mating isolation (Table 1-1). Critically, it is important to note that the absence of hybrids

does not indicate that hybrid matings do not occur in the wild. Reproductive interaction between species may still occur and lead to wasted gametes (Hochkirch et al., 2007). Eggs fertilized by a non-conspecific male can not be unfertilized and still represent evolutionary dead ends even if hybrids are not produced. On top of the species composition of the parents, factors such as the direction of hybridization can also impact hybrid offspring.

The direction of hybridization between species plays a large role in the development of salmonid hybrids (Buss & Wright, 1958; Chevassus, 1979). In crosses between species such as the brook char and brown trout, hybrid direction determines the viability of the offspring in terms of mortality before feeding (Buss & Wright, 1958; Chevassus, 1979). A similar effect has been observed in European hybrids of Atlantic salmon and brown trout. One direction of hybridization (Atlantic salmon mother with a brown trout father) produces largely viable offspring, while the reciprocal direction leads to higher mortality during the last stages of development and an increased incidence of developmental deformities after hatch (Álvarez & Garcia-Vazquez, 2011). This cross's reciprocal direction is the typical direction of wild hybridization for Atlantic salmon and brown trout in Newfoundland, and brown trout eggs fertilized by salmon sperm exhibit greater survival after hatch when compared to the typical European cross (McGowan & Davidson, 1992b; Poulos, 2019).

In aquaculture settings, crosses between Atlantic salmon and brown trout of either sex result in offspring with high survival to one year and reproductively viable gametes (Buss & Wright, 1958). Atlantic salmon x brown trout crosses differ in development times compared to pure-species crosses. Hybrid offspring from a female Atlantic salmon and a male brown trout hatch 5-to-6-degree days earlier than salmon eggs fertilized with Atlantic salmon sperm. These

fish are also larger at hatch than both pure-species crosses (McGowan & Davidson, 1992a). The reciprocal cross tends to be less successful and has a lower hatch rate and higher mortality. Even though I may observe changes in patterns between the study populations linked to the direction of fertilization, sperm exposed to conspecific vs. heterospecific ovarian fluid might react and influence early development differently through gamete-mediated paternal effects because of the alteration of sperm swimming behavior. In this thesis, I seek to examine both questions in the following two chapters.

Thesis organization

The second chapter explores the role that cryptic female choice plays in preventing hybridization using three species. I use Newfoundland populations to partially replicate the sperm behavior portion of a study done with native European Atlantic salmon and brown trout (Yeates et al., 2013) but add another species, brook char, into the experiment. I hope to determine that there is scaling in the strength of this mechanism over three hybridizing species and expect to find a pattern that is linked to taxonomic distance or potential spawning overlap. I posit that conspecific sperm preference is stronger in situations where fish are more closely related and more likely to spawn with one another, for example, in congeneric hybrids vs. heterogeneric hybrids. In chapter 2, I hypothesize that 1) sperm swimming behavior is upregulated by ovarian fluid of any type compared to swimming in just water, 2) ovarian fluid upregulates conspecific sperm more than heterospecific sperm, and 3) there is a pattern in upregulation based on phylogenetic relatedness or likelihood of spawning occurring.

While the mechanism of cryptic female choice and its potential effects on offspring through gamete-mediated paternal effects has not yet been examined, I follow previous work done with Atlantic salmon to examine this potential link. In chapter 3, I examine the consequences of sperm environmental variation on sperm experiences, specifically the aforementioned female barriers to hybridization, on time to hatch and offspring development. In Atlantic salmon, it is reported that sperm that swim longer in water cause offspring to hatch earlier and larger than sperm that fertilize eggs immediately after activation (Immler et al., 2014). Given that sperm swimming could affect offspring so significantly, I sought to replicate this study in the context of hybridization with brown trout. I follow similar protocols as the Immler work but also test for the effects of swimming in conspecific and heterospecific ovarian fluid on both Atlantic salmon and Atlantic salmon brown trout hybrids. Following previous research with these species, it is known that hybrids hatch earlier and larger than pure species crosses (McGowan & Davidson, 1992a; Poulos, 2019); however, it is unknown if paternal effects vary in hybrids or if they are even relevant when compared to effects of hybridization. I hypothesize that offspring development differs depending on whether sperm swim in conspecific or heterospecific ovarian fluid, in ovarian fluid or water, or for 20 seconds or 0 seconds (fertilization immediately after activation). I also hypothesize that the effects of hybridization on offspring are greater than paternal effects and that hybrids are affected the same as Atlantic salmon.

Co-authorship statement

Tyler Lantiegne played a key role in the design and planning of the two experiments. He carried out the data collection, processed and analyzed the data, and wrote the subsequent chapters.

Dr. Craig Purchase conceived the studies and provided substantial contributions to experimental design, fieldwork, and statistical analysis, and helped to edit and review all thesis chapters. Chapters 2 and 3 are draft manuscripts and thus the language reflects this with the pronoun we. Chapters 1 and 4 were written for this thesis and as such use the pronoun I.

Publication and submission status:

Chapter 2: Lantiegne T. and Purchase C. Cryptic female choice has limited ability to prevent invasive hybridization in externally fertilizing fish. This manuscript is planned to be published and will be prepared for submission to a peer-reviewed journal.

Chapter 3: Lantiegne T. and Purchase C. Cryptic female choice does not alter gamete-mediated paternal effects in hybridizing fish. This manuscript is planned to be published and will be prepared for submission to a peer-reviewed journal.

*Appendices are included for both chapters for thesis examination but are not planned to be included with the chapters when they are published.

Literature cited

Abbott, R., Albach, D., Ansell, S., Arntzen, J. W., Baird, S. J. E., Bierne, N., Boughman, J., Brelsford, A., Buerkle, C. A., Buggs, R., Butlin, R. K., Dieckmann, U., Eroukhmanoff, F., Grill, A., Cahan, S. H., Hermansen, J. S., Hewitt, G., Hudson, A. G., Jiggins, C., … Zinner, D. (2013). Hybridization and speciation. *Journal of Evolutionary Biology*, *26*(2), 229–246. https://doi.org/10.1111/j.1420-9101.2012.02599.x

Albo, M., Toft, S., & Bilde, T. (2014). Sexual Selection, ecology, and evolution of nuptial gifts in spiders. In *Sexual Selection: Perspectives and Models from the Neotropics* (pp. 183–200). https://doi.org/10.1016/B978-0-12-416028-6.00007-4

Alonzo, S. H., Stiver, K. A., & Marsh-Rollo, S. E. (2016). Ovarian fluid allows directional cryptic female choice despite external fertilization. *Nature Communications*, *7*(1), 12452. https://doi.org/10.1038/ncomms12452

Álvarez, D., & Garcia-Vazquez, E. (2011). Maintenance of asymmetric hybridization between Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) via postzygotic barriers and paternal effects. *Canadian Journal of Fisheries and Aquatic Sciences, 68*(4), 593-602. https://doi.org/10.1139/f2011-004

Andersson, M. (1994). *Sexual Selection* (2nd ed.). https://press.princeton.edu/books/paperback/9780691000572/sexual-selection

Auld, H. L., Noakes, D. L., & Banks, M. (2019). Advancing mate choice studies in salmonids. *Reviews in Fish Biology and Fisheries*. https://doi.org/10.1007/s11160-019-09551-5

Bagenal, T. B. (1969). Relationship between egg size and fry survival in brown trout (*Salmo trutta*) L. *Journal of Fish Biology*, *1*(4), 349–353. https://doi.org/10.1111/j.1095-8649.1969.tb03882.x

Basolo, A. L. (1990). Female preference predates the evolution of the sword in swordtail fish. *Science (New York, N.Y.)*, *250*(4982), 808–810. https://doi.org/10.1126/science.250.4982.808

Bateman, A. J. (1948). Intra-sexual selection in *Drosophila*. *Heredity*, *2*(3), 349–368. https://doi.org/10.1038/hdy.1948.21

Bernardo, J. (1996). Maternal effects in animal ecology. *American Zoologist*, *36*(2), 83–105. https://doi.org/10.1093/icb/36.2.83

Birkhead, Timothy R., & Pizzari, T. (2002). Postcopulatory sexual selection. *Nature Reviews. Genetics*, *3*(4), 262–273. https://doi.org/10.1038/nrg774

Blanc, J. M., & Chevassus, B. (1979). Interspecific hybridization of salmonid fish. *Aquaculture*, *18*(1), 21–34. https://doi.org/10.1016/0044-8486(79)90097-8

Buss, K., & Wright, J. E. (1958). Appearance and fertility of trout Hybrids. *Transactions of the American Fisheries Society*, *87*(1), 172–181. https://doi.org/10.1577/1548-8659(1957)87[172: AAFOTH]2.0.CO;2

Castillo, Y., & Arce, E. (2020). Female preference for dominant males in the Mexican mojarra cichlid fish, (*Cichlasoma istlanum*). *Journal of Fish Biology*, *98*(1), 189-195. https://doi.org/10.1111/jfb.14569

Chang, H., Kim, B. J., Kim, Y. S., Suarez, S. S., & Wu, M. (2013). Different migration patterns of sea urchin and mouse sperm revealed by a microfluidic chemotaxis device. *PLoS ONE*, *8*(4). https://doi.org/10.1371/journal.pone.0060587

Chevassus, B. (1979). Hybridization in salmonids: Results and perspectives. *Aquaculture*, *17*(2), 113–128. https://doi.org/10.1016/0044-8486(79)90047-4

Clutton-Brock, T., & McAuliffe, K. (2009). Female mate choice in mammals. *The Quarterly Review of Biology*, *84*(1), 3–27. https://doi.org/10.1086/596461

Cothran, R. D. (2008). Direct and indirect fitness consequences of female choice in a crustacean. *Evolution*, *62*(7), 1666–1675. https://doi.org/10.1111/j.1558-5646.2008.00403.x

Cucherousset, J., Aymes, J. C., Poulet, N., Santoul, F., & Céréghino, R. (2008). Do native brown trout and non-native brook trout interact reproductively? *Naturwissenschaften*, *95*(7), 647–654. https://doi.org/10.1007/s00114-008-0370-3

Cucherousset, J., Aymes, J. C., Santoul, F., & Céréghino, R. (2007). Stable isotope evidence of trophic interactions between introduced brook trout (*Salvelinus fontinalis*) and native brown trout (*Salmo trutta*) in a mountain stream of south-west France. *Journal of Fish Biology*, *71*(sd), 210–223. https://doi.org/10.1111/j.1095-8649.2007.01675.x

Cunjak, R. A., & Power, G. (1986). Winter habitat utilization by stream resident brook trout (*Salvelinus fontinalis*) and brown trout *(Salmo trutta*). *Canadian Journal of Fisheries and Aquatic Sciences*, *43*(10), 1970–1981. https://doi.org/10.1139/f86-242

Dangel, Jim., Dangel, J., Macy, P. T., & Withler, F. C. (1973). Annotated bibliography of interspecific hybridization of fishes of the subfamily Salmoninae (pp. 1–56). U.S. Dept. of

Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service; https://doi.org/10.5962/bhl.title.61757

Darwin, C. (1871). The descent of man: And selection in relation to sex. London: J. Murray.

Donkin, I., & Barrès, R. (2018). Sperm epigenetics and influence of environmental factors. *Molecular Metabolism*, *14*, 1–11. https://doi.org/10.1016/j.molmet.2018.02.006

Edward, D. A., Stockley, P., & Hosken, D. J. (2015). Sexual conflict and sperm competition. *Cold Spring Harbor Perspectives in Biology*, *7*(4), a017707.. https://doi.org/10.1101/cshperspect.a017707

Eilertsen, E., Bårdsen, B.-J., Liljedal, S., Rudolfsen, G., & Folstad, I. (2008). Experimental evidence for paternal effects on offspring growth rate in Arctic charr (*Salvelinus alpinus*). *Proceedings of the Royal Society B: Biological Science*, *276*, 129–136. https://doi.org/10.1098/rspb.2008.0884

Einum, S., & Fleming, I. (1999). Einum S, Fleming IA.. Maternal effects of egg size in brown trout (*Salmo trutta*): Norms of reaction to environmental quality. *Proceedings of the Royal Society B: Biological Sciences*, *266*, 1995–2000. https://doi.org/10.1098/rspb.1999.0893

Emery Thompson, M., & Georgiev, A. V. (2014). The high price of success: Costs of mating effort in male primates. *International Journal of Primatology*, *35*(3), 609–627. https://doi.org/10.1007/s10764-014-9790-4

Esteve, M. (2005). Observations of spawning behaviour in Salmoninae: *Salmo*, *Oncorhynchus* and *Salvelinus*. *Reviews in Fish Biology and Fisheries*, *15*, 1–21. https://doi.org/10.1007/s11160-005-7434-7

Evans, J. P., Pierotti, M., & Pilastro, A. (2003). Male mating behavior and ejaculate expenditure under sperm competition risk in the eastern mosquitofish. *Behavioral Ecology*, *14*(2), 268–273. https://doi.org/10.1093/beheco/14.2.268

Evans, J. P., Wilson, A. J., Pilastro, A., & Garcia-Gonzalez, F. (2019). Ejaculate-mediated paternal effects: Evidence, mechanisms, and evolutionary implications. *Reproduction*, *157*(4), R109–R126. https://doi.org/10.1530/REP-18-0524

Fausch, K. D., & White, R. J. (1981). Competition between brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*) for positions in a Michigan stream. *Canadian Journal of Fisheries and Aquatic Sciences*, *38*(10), 1220–1227. https://doi.org/10.1139/f81-164

Firman, R. C., Gasparini, C., Manier, M. K., & Pizzari, T. (2017). Postmating female control: 20 years of cryptic female choice. *Trends in Ecology & Evolution*, *32*(5), 368–382. https://doi.org/10.1016/j.tree.2017.02.010

Fisher, R. A. (1915). The evolution of sexual preference. *The Eugenics Review*, *7*(3), 184–192.

Friesen, C. R., Uhrig, E. J., Mason, R. T., & Brennan, P. L. R. (2016). Female behaviour and the interaction of male and female genital traits mediate sperm transfer during mating. *Journal of Evolutionary Biology*, *29*(5), 952–964. https://doi.org/10.1111/jeb.12836

Froy, H., Walling, C. A., Pemberton, J. M., Clutton-Brock, T. H., & Kruuk, L. E. B. (2016). Relative costs of offspring sex and offspring survival in a polygynous mammal. *Biology Letters*, *12*(9). https://doi.org/10.1098/rsbl.2016.0417

Galimberti, F., Boitani, L., & Marzetti, I. (2000). Female strategies of harassment reduction in southern elephant seals. *Ethology Ecology & Evolution*, *12*(4), 367–388. https://doi.org/10.1080/08927014.2000.9522793

Garcia-Gonzalez, F., & Simmons, L. (2005). The evolution of polyandry: Intrinsic sire effects contribute to embryo viability. *Journal of Evolutionary Biology*, *18*(4), 1097-1103 https://doi.org/10.1111/j.1420-9101.2005.00889.x

Garcia-Vazquez, E., Moran, P., Perez, J., Martinez, J. L., Izquierdo, J. I., de Gaudemar, B., & Beall, E. (2002). Interspecific barriers between salmonids when hybridisation is due to sneak mating. *Heredity*, *89*(4), 288–292. https://doi.org/10.1038/sj.hdy.6800134

Garcia-Vazquez, Eva, Perez, J., Ayllon, F., Martinez, J. L., Glise, S., & Beall, E. (2004). Asymmetry of post-F1 interspecific reproductive barriers among brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*). *Aquaculture*, *234*(1), 77–84. https://doi.org/10.1016/j.aquaculture.2004.01.017

Gasparini, C., Lu, C., Dingemanse, N. J., & Tuni, C. (2018). Paternal effects in a terrestrial ectotherm are temperature dependent but no evidence for adaptive effects. *Functional Ecology*, *32*(4), 1011–1021. https://doi.org/10.1111/1365-2435.13022

Gasparini, C., & Pilastro, A. (2011). Cryptic female preference for genetically unrelated males is mediated by ovarian fluid in the guppy. *Proceedings of the Royal Society B: Biological Sciences*, *278*(1717), 2495–2501. https://doi.org/10.1098/rspb.2010.2369

Gasparini, C., Pilastro, A., & Evans, J. P. (2020). The role of female reproductive fluid in sperm competition. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *375*(1813), 20200077. https://doi.org/10.1098/rstb.2020.0077

Gergus, E. W. A., Malmos, K. B., & Sullivan, B. (1999). Natural hybridization among distantly related toads (*Bufo alvarius, Bufo cognatus, Bufo woodhousii*) in central Arizona. *Copeia*, *2*, 281–286. https://doi.org/10.2307/1447473

Gibson, R.J., J. Hammar, and G. Mitchell. 1999. The Star Lake hydroelectric project – an example of the failure of the Canadian Environmental Assessment Act. p. 147-176. *In*: Ryan, P.M. (ed.) Assessments and impacts of megaprojects. Proceedings of the 38th Annual Meeting of the Canadian Society of Environmental Biologists in collaboration with the Newfoundland and Labrador Environment Network, St. John's, Nfld. Canada, October 1-3, 1998. Canadian Society of Environmental Biologists. Toronto. X + 233 pp.

Gowaty, P. A., Kim, Y.-K., Rawlings, J., & Anderson, W. W. (2010). Polyandry increases offspring viability and mother productivity but does not decrease mother survival in *Drosophila pseudoobscura*. *Proceedings of the National Academy of Sciences*, *107*(31), 13771–13776. https://doi.org/10.1073/pnas.1006174107

Goymann, W., Safari, I., Muck, C., & Schwabl, I. (2016). Sex roles, parental care, and offspring growth in two contrasting coucal species. *Royal Society Open Science*, *3*(10), 160463. https://doi.org/10.1098/rsos.160463

Grant, P. R., & Grant, B. R. (1997). Hybridization, sexual imprinting, and mate choice. *The American Naturalist*, *149*(1), 1–28.

Gross, M. R. (1996). Alternative reproductive strategies and tactics: Diversity within sexes. *Trends in Ecology & Evolution*, *11*(2), 92–98. https://doi.org/10.1016/0169-5347(96)81050-0

Harrison, R. G., & Larson, E. L. (2014). Hybridization, introgression, and the nature of species boundaries. *Journal of Heredity*, *105*(S1), 795–809. https://doi.org/10.1093/jhered/esu033

Hartley, S. E. (1987). The chromosomes of salmonid fishes. *Biological Reviews*, *62*(3), 197–214. https://doi.org/10.1111/j.1469-185X.1987.tb00663.x

Hayward, A., & Gillooly, J. F. (2011). The cost of sex: Quantifying energetic investment in gamete production by males and females. *PLoS ONE*, *6*(1), e16557. https://doi.org/10.1371/journal.pone.0016557

Head, M. L., Hunt, J., Jennions, M. D., & Brooks, R. (2005). The indirect benefits of mating with attractive males outweigh the direct costs. *PLoS Biology*, *3*(2), e33. https://doi.org/10.1371/journal.pbio.0030033

Heath, D. D., Fox, C. W., & Heath, J. W. (1999). Maternal effects on offspring size: Variation through early development of chinook salmon. *Evolution*, *53*(5), 1605–1611. https://doi.org/10.2307/2640906

Heggberget, T. G., Haukebø, T., Mork, J., & Ståhl, G. (1988). Temporal and spatial segregation of spawning in sympatric populations of Atlantic salmon, (*Salmo salar*), and brown trout, (*Salmo trutta*) *Journal of Fish Biology*, *33*(3), 347–356. https://doi.org/10.1111/j.1095-8649.1988.tb05477.x

Hendry, A., P., & Stearns, Stephen C. (2003). Evolution illuminated: Salmon and their relatives. Oxford University Press.

Hochkirch, A., Gröning, J., & Bücker, A. (2007). Sympatry with the devil: Reproductive interference could hamper species coexistence. *Journal of Animal Ecology*, *76*(4), 633–642. https://doi.org/10.1111/j.1365-2656.2007.01241.x

Holman, L., & Snook, R. R. (2008). A sterile sperm caste protects brother fertile sperm from female-mediated death in *Drosophila pseudoobscura*. *Current Biology: CB*, *18*(4), 292–296. https://doi.org/10.1016/j.cub.2008.01.048

Hosken, D. J., Garner, T. w. J., Tregenza, T., Wedell, N., & Ward, P. I. (2003). Superior sperm competitors sire higher–quality young. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1527), 1933–1938. https://doi.org/10.1098/rspb.2003.2443

Howard, D. J., Palumbi, S. R., Birge, L. M., & Manier, M. K. (2009). 9—Sperm and speciation. In Tim R. Birkhead, D. J. Hosken, & S. Pitnick (Eds.), *Sperm Biology* (pp. 367–403). Academic Press. https://doi.org/10.1016/B978-0-12-372568-4.00009-4

Hubbs, C. L. (1955). Hybridization between fish species in nature. *Systematic Biology*, *4*(1), 1–20. https://doi.org/10.2307/sysbio/4.1.1

Hustins, D. (2007). Brown Trout and Rainbow Trout: A Journey Into Newfoundland Waters. Tight Lines Publishers.

Immler, S. (2018). The sperm factor: Paternal impact beyond genes. *Heredity*, *121*(3), 239–247. https://doi.org/10.1038/s41437-018-0111-0

Immler, S., Hotzy, C., Alavioon, G., Petersson, E., & Arnqvist, G. (2014). Sperm variation within a single ejaculate affects offspring development in Atlantic salmon. *Biology Letters*, *10*(2), 20131040. https://doi.org/10.1098/rsbl.2013.1040

Ito, D., Fujiwara, A., & Abe, S. (2006). Hybrid inviability and chromosome abnormality in salmonid fish. *The Journal of Animal Genetics*, *34*(1), 65–70. https://doi.org/10.5924/abgri2000.34.65

Jansson, H., Holmgren, I., Wedin, K., & Anderson, T. (1991). High frequency of natural hybrids between Atlantic salmon, (*Salmo salar*), and brown trout, (*Salmo trutta*), in a Swedish river. *Journal of Fish Biology*, *39*(sA), 343–348. https://doi.org/10.1111/j.1095-8649.1991.tb05096.x

Jones, A. G., & Ratterman, N. L. (2009). Mate choice and sexual selection: What have we learned since Darwin? *Proceedings of the National Academy of Sciences*, *106*(Supplement 1), 10001–10008. https://doi.org/10.1073/pnas.0901129106

Kamler, E. (2005). Parent–egg–progeny relationships in teleost fishes: An energetics perspective. *Reviews in Fish Biology and Fisheries*, *15*(4), 399. https://doi.org/10.1007/s11160-006-0002-y

Kappeler, L., & Meaney, M. J. (2010). Epigenetics and parental effects. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*, *32*(9), 818–827. https://doi.org/10.1002/bies.201000015

Klemme, I., Ylönen, H., & Eccard, J. A. (2008). Long-term fitness benefits of polyandry in a small mammal, the bank vole *Clethrionomys glareolus*. *Proceedings of the Royal Society B: Biological Sciences*, *275*(1638), 1095–1100. https://doi.org/10.1098/rspb.2008.0038

Klibansky, L. K. J., & McCartney, M. A. (2014). Conspecific sperm precedence is a reproductive barrier between free-spawning marine mussels in the northwest Atlantic *Mytilus* hybrid zone. *PLoS ONE*, *9*(9), e108433. https://doi.org/10.1371/journal.pone.0108433

Kokko, H., & Jennions, M. D. (2008). Parental investment, sexual selection, and sex ratios. *Journal of Evolutionary Biology*, *21*(4), 919–948. https://doi.org/10.1111/j.1420-9101.2008.01540.x

Kristjánsson, L. T., & Vøllestad, L. A. (1996). Individual variation in progeny size and quality in rainbow trout, (*Oncorhynchus mykiss*). *Aquaculture Research*, *27*(5), 335–343. https://doi.org/10.1111/j.1365-2109.1996.tb01260.x

Krueger, C. C., & May, B. (1991). Ecological and genetic effects of salmonid introductions in North America. *Canadian Journal of Fisheries and Aquatic Sciences, 48*(s1), 66-77. https://doi.org/10.1139/f91-305

Kuijper, B., Pen, I., & Weissing, F. J. (2012). A guide to sexual selection theory. *Annual Review of Ecology, Evolution, and Systematics*, *43*(1), 287–311. https://doi.org/10.1146/annurev-ecolsys-110411-160245

Lecaudey L.A., Schliewen U.K., Osinov A.G., Taylor E.B., Bernatchez L. & Weiss S.J. (2018) Inferring phylogenetic structure, hybridization and divergence times within Salmoninae (Teleostei: Salmonidae) using RAD sequencing. *Molecular Phylogenetics and Evolution* **124**, 82–99.

Lehnert, Sarah J., Kess, T., Bentzen, P., Clément, M., & Bradbury, I. R. (2020). Divergent and linked selection shape patterns of genomic differentiation between European and North American Atlantic salmon (*Salmo salar*). *Molecular Ecology*, *29*(12), 2160–2175. https://doi.org/10.1111/mec.15480

Lymbery, R. A., Kennington, W. J., & Evans, J. P. (2017). Egg chemoattractants moderate intraspecific sperm competition. *Evolution Letters*, *1*(6), 317–327. https://doi.org/10.1002/evl3.34

MacCrimmon, H. R., & Marshall, T. L. (1968). World Distribution of Brown Trout, Salmo trutta. *Journal of the Fisheries Board of Canada*. https://doi.org/10.1139/f68-225

Maheshwari, S., & Barbash, D. A. (2011). The genetics of hybrid incompatibilities. *Annual Review of Genetics*, *45*(1), 331–355. https://doi.org/10.1146/annurev-genet-110410-132514

Makhrov, Alexander. (2008). Makhrov A.A. 2008. Hybridization of the Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*). *Zoosystematica Rossica*, *17*(2), 129-143. https://doi.org/10.31610/zsr/2008.17.2.129

Marshall, D. J., & Bolton, T. F. (2007). Sperm release strategies in marine broadcast spawners: The costs of releasing sperm quickly. *Journal of Experimental Biology*, *210*(21), 3720–3727. https://doi.org/10.1242/jeb.008417

Mason, R., & Crews, D. (1985). Female mimicry in garter snakes. *Nature*, *316*, 59–60. https://doi.org/10.1038/316059a0

McGowan, C., & Davidson, W. S. (1992a). Artificial hybridization of Newfoundland brown trout and Atlantic salmon: Hatchability, survival, and growth to first feeding. *Aquaculture*, *106*(2), 117–125. https://doi.org/10.1016/0044-8486(92)90196-R

McGowan, C., & Davidson, W. S. (1992b). Unidirectional natural hybridization between brown trout (*Salmo trutta*) and Atlantic salmon (*S*almo *salar*) in Newfoundland. *Canadian Journal of Fisheries and Aquatic Sciences*, *49*(9), 1953–1958. https://doi.org/10.1139/f92-216

Mousseau, T. A., & Fox, C. W. (1998). The adaptive significance of maternal effects. *Trends in Ecology & Evolution*, *13*(10), 403–407. https://doi.org/10.1016/s0169-5347(98)01472-4

Muldal, A. M., Moffatt, J. D., & Robertson, R. J. (1986). Parental care of nestlings by male red-winged blackbirds. *Behavioral Ecology and Sociobiology*, *19*(2), 105–114. https://doi.org/10.1007/BF00299945

Neat, F. C. (2001). Male parasitic spawning in two species of triplefin blenny (Tripterigiidae): contrasts in demography, behaviour and gonadal characteristics. *Environmental Biology of Fishes*, *61*(1), 57–64. https://doi.org/10.1023/A:1011074716758

Nomakuchi, S., & Higashi, K. (1996). Competitive habitat utilization in the damselfly, *Mnais nawai* (Zygoptera: Calopterygidae) coexisting with a related species, *Mnais pruinosa*. *Researches on Population Ecology*, *38*(1), 41–50. https://doi.org/10.1007/BF02514969

Norman, M. D., Finn, J., & Tregenza, T. (1999). Female impersonation as an alternative reproductive strategy in giant cuttlefish. *Proceedings of the Royal Society B: Biological Sciences*, *266*(1426), 1347–1349.

Nygren, A., Nilsson, B., & Jahnke, M. (1972). Cytological studies in Atlantic salmon from Canada, in hybrids between Atlantic salmon from Canada and Sweden and in hybrids between Atlantic salmon and sea trout. *Hereditas*, *70*(2), 295–305. https://doi.org/10.1111/j.1601-5223.1972.tb01388.x

O'Connell, M. F. (1982). The biology of anadromous *Salvelinus fontinalis* (Mitchill, 1815*)* and *Salmo trutta* (Linnaeus, 1758) in river systems flowing into Placentia Bay and St. Mary's Bay, Newfoundland [Doctoral, Memorial University of Newfoundland]. https://research.library.mun.ca/4191/

Ojanguren, A. F., Reyes-Gavilán, F. G., & Braña, F. (1996). Effects of egg size on offspring development and fitness in brown trout, (*Salmo trutta) Aquaculture*, *147*(1), 9–20. https://doi.org/10.1016/S0044-8486(96)01398-1

Ortíz-Barrientos, D., Counterman, B. A., & Noor, M. A. F. (2007). Gene expression divergence and the origin of hybrid dysfunctions. *Genetica*, *129*(1), 71–81. https://doi.org/10.1007/s10709-006-0034-1

Page, L. M., Burr, B. M., Society, N. A., Federation, N. W., & Institute, R. T. P. (1991). A field guide to freshwater fishes: North America north of Mexico. Houghton Mifflin.

Parker, G. A. (1970). Sperm competition and its evolutionary consequences in the insects. *Biological Reviews*, *45*(4), 525–567. https://doi.org/10.1111/j.1469-185X.1970.tb01176.x

Parker, G. A., & Partridge, L. (1998). Sexual conflict and speciation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *353*(1366), 261–274. https://doi.org/10.1098/rstb.1998.0208

Parker, G. A. (2014). The sexual cascade and the rise of pre-ejaculatory (Darwinian) sexual selection, sex roles, and sexual conflict. *Cold Spring Harbor Perspectives in Biology*, *6*(10), a017509. https://doi.org/10.1101/cshperspect.a017509

Penney, H. D., Beirão, J., & Purchase, C. F. (2018). Phenotypic plasticity during external embryonic development is affected more by maternal effects than multiple abiotic factors in brook trout. *Evolutionary Ecology Research*, *19*(2), 171–194.

Pianka, E. R. (1970). On r- and K-Selection. *The American Naturalist*, *104*(940), 592–597.

Pizzari, T., & Birkhead, T. R. (2000). Female feral fowl eject sperm of subdominant males. *Nature*, *405*(6788), 787–789. https://doi.org/10.1038/35015558

Poli, F., Immler, S., & Gasparini, C. (2019). Effects of ovarian fluid on sperm traits and its implications for cryptic female choice in zebrafish. *Behavioral Ecology*, *30*(5), 1298–1305. https://doi.org/10.1093/beheco/arz077

Poulos, S. (2019). Embryo development under context of hybridization and a review of regional patterns in hybrid frequency of Atlantic salmon and brown trout [Masters, Memorial University of Newfoundland]. https://research.library.mun.ca/13969/

Préault, M., Chastel, O., Cézilly, F., & Faivre, B. (2005). Male bill colour and age are associated with parental abilities and breeding performance in blackbirds. *Behavioral Ecology and Sociobiology*, *58*, 497–505. https://doi.org/10.1007/s00265-005-0937-3

Qvarnström, A., & Price, T. D. (2001). Maternal effects, paternal effects and sexual selection. *Trends in Ecology & Evolution*, *16*(2), 95–100. https://doi.org/10.1016/S0169-5347(00)02063-2

Régnier, T., Bolliet, V., Labonne, J., & Gaudin, P. (2010). Assessing maternal effects on metabolic rate dynamics along early development in brown trout (*Salmo trutta*): An individual-based approach. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology*, *180*(1), 25–31. https://doi.org/10.1007/s00360-009-0385-x

Ritchie, H., & Marshall, D. J. (2013). Fertilisation is not a new beginning: Sperm environment affects offspring developmental success. *Journal of Experimental Biology*, *216*(16), 3104–3109. https://doi.org/10.1242/jeb.087221

Robertson, S. A. (2007). Seminal fluid signaling in the female reproductive tract: Lessons from rodents and pigs. *Journal of Animal Science*, *85*(13 Suppl), E36-44. https://doi.org/10.2527/jas.2006-578

Ryan, M. J. (1980). Female mate choice in a neotropical frog. *Science*, *209*(4455), 523–525. https://doi.org/10.1126/science.209.4455.523

Ryan, M. J. (1998). Sexual selection, receiver biases, and the evolution of sex differences. *Science*, *281*(5385), 1999–2003. https://doi.org/10.1126/science.281.5385.1999

Schatten, H., & Sun, Q.-Y. (2010). The role of centrosomes in fertilization, cell division and establishment of asymmetry during embryo development. *Seminars in Cell & Developmental Biology*, *21*(2), 174–184. https://doi.org/10.1016/j.semcdb.2010.01.012

Schnakenberg, S. L., Siegal, M. L., & Bloch Qazi, M. C. (2012). Oh, the places they'll go: Female sperm storage and sperm precedence in *Drosophila melanogaster*. *Spermatogenesis*, *2*(3), 224–235. https://doi.org/10.4161/spmg.21655

Schwenk, K., Brede, N., & Streit, B. (2008). Introduction. Extent, processes and evolutionary impact of interspecific hybridization in animals. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1505), 2805–2811. https://doi.org/10.1098/rstb.2008.0055

Shuster, S. M. (2009). Sexual selection and mating systems. *Proceedings of the National Academy of Sciences*, *106*(Supplement 1), 10009–10016. https://doi.org/10.1073/pnas.0901132106

Sorensen, P. W., Essington, T., Weigel, D. E., & Cardwell, J. R. (1995). Reproductive interactions between sympatric brook and brown trout in a small Minnesota stream. *Canadian*

Journal of Fisheries and Aquatic Sciences, *52*(9), 1958–1965. https://doi.org/10.1139/f95-787

Sutterlin, A. M., MacFarlane, L. R., & Harmon, P. (1977). Growth and salinity tolerance in hybrids within *Salmo sp*. And *Salvelinus sp*. *Aquaculture*, *12*(1), 41–52. https://doi.org/10.1016/0044-8486(77)90045-X

Trivers, R. L. (1972). Sexual election and the Descent of Man, 1871-1971. Aldine, Chicago.

Troisi, A., & Carosi, M. (1998). Female orgasm rate increases with male dominance in Japanese macaques. *Animal Behaviour*, *56*(5), 1261–1266. https://doi.org/10.1006/anbe.1998.0898

Verspoor, E. (1988). Widespread hybridization between native Atlantic salmon, (*Salmo salar*), and introduced brown trout, (*Salmo trutta*), in eastern Newfoundland. *Journal of Fish Biology*, *32*(3), 327–334. https://doi.org/10.1111/j.1095-8649.1988.tb05370.x

Vladić, T., Forsberg, L. A., & Järvi, T. (2010). Sperm competition between alternative reproductive tactics of the Atlantic salmon in vitro. *Aquaculture*, *302*(3), 265–269. https://doi.org/10.1016/j.aquaculture.2010.02.024

Wedekind, C., Jacob, A., Evanno, G., Nusslé, S., & Müller, R. (2008). Viability of brown trout embryos positively linked to melanin-based but negatively to carotenoid-based colours of their fathers. *Proceedings of the Royal Society B: Biological Sciences*, *275*(1644), 1737–1744. https://doi.org/10.1098/rspb.2008.0072

Westley, P. A. H., & Fleming, I. A. (2011). Landscape factors that shape a slow and persistent aquatic invasion: Brown trout in Newfoundland 1883–2010. *Diversity and Distributions*, *17*(3), 566–579. https://doi.org/10.1111/j.1472-4642.2011.00751.x

Willis, P. M. (2013). Why do animals hybridize? *Acta Ethologica*, *16*(3), 127–134. https://doi.org/10.1007/s10211-013-0144-6

Xu, J., & Wang, Q. (2010). Mechanisms of last male precedence in a moth: Sperm displacement at ejaculation and storage sites. *Behavioral Ecology*, *21*(4), 714–721. https://doi.org/10.1093/beheco/arq044

Yeates, S. E., Diamond, S. E., Einum, S., Emerson, B. C., Holt, W. V., & Gage, M. J. G. (2013). Cryptic choice of conspecific sperm controlled by the impact of ovarian fluid on sperm swimming behavior. *Evolution*, *67*(12), 3523–3536. https://doi.org/10.1111/evo.12208

Young, B., Conti, D. V., & Dean, M. D. (2013). Sneaker "jack" males outcompete dominant "hooknose" males under sperm competition in chinook salmon (*Oncorhynchus tshawytscha*). *Ecology and Evolution*, *3*(15), 4987–4997. https://doi.org/10.1002/ece3.869

Chapter 1 tables

Table 1-1: Summary of costs of phylogenetic relatedness, the likelihood of egg exposure, and hybrid fitness. Bold and italicized text indicates the author's original conclusions. References: 1 (Chevassus, 1979), 2 (Makhrov, 2008), 3 (McGowan and Davidson, 1992b), 4 (Sorenson et al., 1995), 5 (O'Connell, 1982), 6 (Lecaudey et al., 2018), 7 (Sutterlin et al., 1977), 8 (Nygren et al., 1972) 9 (Hartley, 1987), 10 (Buss and Wright, 1958), 11 (Garcia-Vasquez et al., 2004), 12 (Blanc and Chevassus, 1979).

Hybrid (mother species/ father species)	Salmon/Trout	Trout/Salmon	Trout/Char	Char/Trout	Salmon/Char	Char/Salmon
Taxonomic relatedness	• **Close** • Same genus	• **Close** • Same genus	• **Most distant** • Different genus • Share less recent common ancestor[6]	• **Most distant** • Different genus • Share less recent common ancestor[6]	• **Moderately distant** • Different genus • Share more recent common ancestor[6]	• **Moderately distant** • Different genus • Share more recent common ancestor[6]
Spawning overlap	• **Very high** • Both species observed to spawn at same place and time[3]	• **Very high** • Both species observed to spawn at the same place and time[3]	• **Very high** • Overlap in spawning period and habitat[4]	• **Very high** • Overlap in spawning period and habitat[4]	• **Medium, brook char males may still be active during salmon spawning** • Small overlap[3,5]	• **High, Atlantic salmon sneaker males may be active during brook char spawning** • Small overlap[3,5]
Likelihood of eggs exposed to sperm	• **High** • Both species observed to spawn with each other[3]	• **High** • Both species observed to spawn with each other[3]	• **High** • Both species observed to spawn with each other[4]	• **High** • Both species observed to spawn with each other[4]	• **Low, brook char males might still be active during salmon spawning.** • Potential brook char sneakers, not known for sure[4]	• **Medium, brook char females unlikely to be ripe during peak salmon spawning** • **Early Atlantic salmon sneakers may try to fertilize eggs**
Fertilization occurs	• Yes[1]	• Yes[1]	• Yes[1]	• Yes[1]	• Yes[1]	• Yes[7]
Parent/hybrid chromosome numbers (North American numbers)	• Mother 58[8] • Father 80[8] • Hybrid 69[8]	• Mother 80[8] • Father 58[8] • Hybrid 69[8]	• Mother 80[8] • Father 84[9] • No data	• Mother 80[8] • Father 84[9] • No data	• Mother 58[8] • Father 84[9] • No data	• Mother 84[9] • Father 58[8] • No data
Offspring hatch	• **Yes, high hatch rate** • 80-100% of the hatch rate of the conspecific cross[1]	• **Yes, high hatch rate** • 80-100% of the hatch rate of the conspecific cross[1]	• **Yes, high hatch rate** • 80-100% of the hatch rate of the conspecific cross[1]	• **Yes, low hatch rate** • <10% of the hatch of the conspecific cross[1]	• **Yes, medium hatch rate** • 40-80% of the hatch rate of the conspecific cross[1]	• **Yes, very low hatch rate** • 3% hatch rate in one case[7] • Total mortality before hatch in another study[12]
Offspring survival to one year	• **F1 high** • 80-100% of the survival of the conspecific cross[1] • F2 none[2]	• **F1 high** • 80-100% of the survival of the conspecific cross[1] • F2 none[2]	• **F1 medium** • 40-80% of the conspecific cross[1]	• **F1 low** • <10 percent of the survival of the conspecific cross[1]	• **F1 low** • <10 percent the survival of the conspecific cross[1]	• None[1]
Offspring fertility	• F1's fertile[1] • Hybrid-hybrid F2's infertile[2] • Backcrosses of this cross to salmon produce offspring with high survival and reduced fecundity[11]	• F1's fertile[1] • Hybrid-hybrid F2's infertile[2] • Backcrosses of this cross to salmon produce infertile offspring with low survival[11]	• F1 fertility extremely rare[10] • Offspring in this case backcrossed with brook trout female resulting in very low survival to fry stage[10]	• F1 infertile[1]	• F1 infertile[1]	• None[1]
Reproductive costs to parents	• **Near total loss by F2 generation**	• **Total loss by F2 generation**	• **Total loss by F1 generation**	• **Total loss by F1 generation**	• **Total loss by F1 generation**	• **Total loss by F1 generation**

Chapter 2

Cryptic female choice has limited ability to prevent invasive hybridization in externally fertilizing fish

Abstract

Polygynandry opens avenues for females to mate with multiple males but also creates opportunities for hybridization through alternative reproductive tactics. Conspecific sperm preference, a process of cryptic female choice, allows females to bias paternity to favor conspecific males in situations of hybridization. This is the first examination of conspecific sperm preference in a system of three species with potential to hybridize, native Atlantic salmon (*Salmo salar*) and brook char (*Salvelinus fontinalis*), and introduced and invasive brown trout (*Salmo trutta*) from three sites in insular Newfoundland. We measured changes in sperm motility and velocity, parameters known to predict paternity, to determine the degree of upregulation to female cues related to conspecific sperm preference. Analyses of differences between sperm behaviour in ovarian fluid and water, conspecific and heterospecific ovarian fluid, and within each ovarian fluid species revealed that all ovarian fluids upregulated sperm motility (53 percent) and velocity (30 percent) compared to water but did not find support for the presence of conspecific sperm preference or patterns of upregulation among species. Subsequently, we conclude that mechanisms of conspecific sperm preference to prevent hybridization are weak in this system and are likely insufficient to promote reproductive isolation, but sperm competition experiments would be needed for confirmation.

Introduction

Sexual selection can occur via intrasex competition between individuals for access to mates and fertilizations, and mate choice for the opposite sex (Andersson, 1994; Jones & Ratterman, 2009; Kuijper et al., 2012). Females are typically the choosier sex and select mates based on various factors, including body odor (Ferkin, 2018), coloration, song, and other courtship displays (Jennions & Petrie, 1997). Males, therefore, usually invest a large amount of energy into creating mating opportunities, while females invest comparatively more energy in the production of gametes and parental care (Bateman, 1948; Trivers, 1972; Emery Thompson & Georgiev, 2014). This difference of energetic expenditure between males and females creates situations where females may benefit from mating polyandrously, with more than one male, to better increase her chances of mating with high-quality males and producing good quality offspring (Firman, 2011).

In polyandrous mating systems, a female's eggs are exposed to sperm from many males, creating sperm competition (Parker, 1970). Polyandry includes situations where females choose mates exhibiting dominant phenotypes (Morina et al., 2018), but other individual males circumvent female choice by resorting to alternative reproductive tactics to sneak fertilizations (Gross, 1996). In some systems, these tactics can result in fertilization by males of a different species, which facilitates hybridization (McGowan & Davidson, 1992; Tynkkynen et al., 2009; Garner & Neff, 2013). Across taxa, hybrid matings can result in highly variable outcomes, including speciation (Abbott et al., 2013), fertile or sterile hybrid offspring (Close & Bell, 1997), or no offspring due to failed fertilization, abortion, or abnormal development (Buss & Wright, 1958; Chevassus, 1979; Wilson et al., 1974). The potential for inviable or sterile offspring

creates energetic waste (Remick, 1992); females have more to lose than males with each hybrid mating and thus should avoid hybrid fertilizations.

Through post-ejaculatory pre-zygotic sexual selection, females can bias sperm competition through cryptic female choice (Firman et al., 2017). The magnitude of this alteration within species can vary based on relatedness (Landry et al., 2001; Yeates et al., 2009), perceived social status (Firman et al., 2017), and quality (Dean et al., 2011) of the male. Mechanisms of cryptic female choice in internal fertilizers include manipulating the duration of copulation, favouring males that provide greater stimulation during copulation, transferring favored sperm to better locations within the reproductive tract, discarding unwanted sperm, removing copulatory plugs, and changing internal conditions to be more or less favorable for sperm (Dixson, 2003; Eberhard, 2010; Pizzari & Birkhead, 2000). External fertilizers do not have this degree of control. Therefore, hybridization is more difficult to avoid in external fertilizers when unchosen males release sperm simultaneously with the female's preferred mate.

However, externally fertilizing females can alter sperm behaviour using chemicals released with eggs, for example, in mussels (Lymbery et al., 2017) and fish (Alonzo et al., 2016; Elofsson et al., 2006). Generally, these chemicals improve sperm swimming performance compared to a water-only environment (Dietrich et al., 2008; Elofsson et al., 2006; Galvano et al., 2013; Lahnsteiner, 2002) and due to differential responses among individuals, subsequently bias fertilizations under sperm competition. Under hybrid matings, this form of cryptic female choice is known as conspecific sperm preference and allows a female to bias fertilization towards her own species (Castillo & Moyle, 2019; Yeates et al., 2013). In studies using paired species, conspecific sperm preference appears to upregulate conspecific sperm swimming

performance more than sperm from heterospecific males (Castillo & Moyle, 2019; Yeates et al., 2013), but how females relatively bias sperm performance in systems containing several species of potential fathers has not been investigated.

How does the strength of cryptic female choice via conspecific sperm preferences vary with multiple species of differing degrees of phylogenetic relatability and/or likelihood of heterospecific matings? A good study system to examine this question is with external fertilizing salmonid fishes, as they are polygynandrous (Haddeland et al., 2015), alternative reproductive tactics via sneak spawning is common (Esteve, 2005), and cryptic female choice mechanisms are reportedly strong (Butts et al., 2012; Rosengrave et al., 2016; Yeates et al., 2013) and readily manipulated. We chose three North American salmonids that can produce hybrids (Chevassus, 1979; Table 1-1), native brook char (*Salvelinus fontinalis*) and Atlantic salmon (*Salmo salar*), and brown trout (*Salmo trutta*), which are introduced and invasive in our study system (Westley & Fleming, 2011). Brown trout create hybrids with both Atlantic salmon (Chevassus, 1979) and brook char (Buss & Wright, 1958) – (Chapter 1), while brook char and Atlantic salmon rarely produce viable offspring as a product of natural mating (Chevassus, 1979) – although mating rates between these species are not known. As a key mechanism to reduce the loss of eggs to hybridization, we hypothesized that (1) any species of ovarian fluid upregulates the swimming performance of any species of sperm when compared to swimming in only water, but (2) that ovarian fluid of all three species upregulates conspecific sperm more than heterospecific sperm, and (3) that there is a pattern in heterospecific upregulation that follows either taxonomic relationships or likelihood of spawning interactions occurring.

Methods

Experimental design

In the presence of sperm competition in natural matings, females are exposed to sperm from multiple males, which creates opportunities to bias paternity. To examine the potential for cryptic female choice, using a split-brood design, we took a sample of ovarian fluid from an individual female, split it into three aliquots, and exposed conspecific and two species of heterospecific sperm to it. We used a split-ejaculate design to quantify the sperm swimming performance in ovarian fluid and a water standard (Figure 2-1) and then determined their ratio. This ratio allowed us to quantify upregulation with a standardized value that is independent of differences between the values (e.g., an increase from 30 to 60 and 40 to 80 gives the same ratio) and thus controls for confounding variables such as individual differences in male quality (Gage et al., 2004; Purchase & Moreau, 2012). A key prediction is that each ovarian fluid species upregulates conspecific sperm more than heterospecific sperm (Figure 2-1).

Sperm swimming performance comparisons were conducted over a series of experimental replicates (unique groups of fish). In each of these replicates, samples of ovarian fluid and sperm from each of our three study species were exposed to one another. Every experimental replicate consisted of 12 sperm swimming performance comparisons between an individual representative from each species of male in water, as well as individual samples of the three ovarian fluids (Table 2-1). For each replicate group of fish, comparisons were technically repeated three times to produce 36 sperm swimming activations. We ran 12 experimental replicates with different fish, with two replicates occurring on a given day. In each replicate, there was one female and one male of each species. In two of the total 36 sampled males, preliminary assessment of semen quality was very poor, and we replaced that fish with

the male from the other replicate on that day. Over the course of the study, we used 70 fish: 12 females of each species, and 12 brown trout, 11 brook char, and 11 Atlantic salmon males. To simplify analyses, we subsequently treated the two reused males as independent, as they were used with different females. We produced 432 sperm swimming comparisons (12 experimental replicates * 3 species of male * 4 sperm activation solutions* 3 procedural replicates).

Fish collection

All fish were sourced from wild populations throughout Newfoundland. Care was taken to ensure that all sperm could be examined within 12 hours of stripping and that population sizes of our study species were large enough to support our study. As a side effect of this, our study fish had differing degrees of allopatry and sympatry. Our brook char and Atlantic salmon had not been exposed to the heterospecific species used in this study. The brown trout we collected had been exposed to brook char, but not Atlantic salmon. Fish and gamete collection for each species varied slightly.

Wild native Atlantic salmon were sourced from the Exploits River in Newfoundland, Canada (48.93 N, 55.67 W). Fish were trapped in the fishway on Grand Falls on September 7, 2018 and transferred to tanks on September 30, following previous protocols (Rooke et al., 2020). At ~11 AM on gamete collection days from November 2 – 14, individuals were anesthetized with MS-222, paper toweled dry, and then stripped of gametes via ventral massage. Semen was stripped into plastic bags and eggs into mason jars.

Wild native brook char were captured via fyke net in the Exploits River watershed at Star Lake in Newfoundland, Canada (48.58 N, 57.23 W) from September 21 to October 5, 2018, transported via truck, and housed in tanks at the same facility as the salmon. Brook char were

fed a diet of mealworms until October 5 and then fed 4mm biobrood pellets for the remaining duration of captivity (salmon do not eat before spawning and were thus not fed). Brook char were anthestized with MS-222. Females were stripped over the last week of October, the eggs were filtered out – see below, and ovarian fluid frozen (Purchase & Rooke, 2020). Brook char males were stripped of gametes immediately after salmon males and females. Fresh char semen and frozen ovarian fluid were stored in 1.5 ml Eppendorf tubes. Gametes from salmon and char were transported on ice and received at the laboratory in St. John's at ~11 PM; all experimental replicates were completed within 24 hours of gamete collection.

There are no brown trout present in the Exploits River watershed from which char and salmon were collected (Westley & Fleming, 2011). Brown trout were introduced from Scotland in the late 19th century (Hustins, 2007) into watersheds surrounding St. John's and have since invaded throughout southeastern Newfoundland. Based on a generation time of three to five years, there have been 27-35 generations of brown trout in Newfoundland. Wild, non-native brown trout used in this study were captured via dipnet in tributaries of Windsor Lake (47.60 N, 52.78 W), in St. John's Newfoundland, where low densities of brook char occur, but there are no Atlantic salmon. Trout were anesthetized with clove oil immediately after capture, measured for length, fin-clipped to avoid double sampling on different days, and stripped for gametes into plastic containers. Through coordinated field activities, trout stripping took place on the same days and at the same time (< one hour) as Atlantic salmon and brook char stripping. Trout gametes were kept on ice for ~12 hours, the same duration as char and salmon before use. Both anesthetics used in this study have been shown to have no significant effects to gametes when used prior to gamete collection (Holcumb et al., 2004).

Gamete preparation

An aliquot of semen (0.5 ml) from each male was centrifuged at 4100 g for 10 minutes at 5°C to separate seminal fluid from sperm. This seminal fluid acted as a non-activating diluting agent to decrease the density of other aliquots (Purchase & Moreau, 2012) at a 1:75 sperm to seminal fluid ratio. This minimized sperm clumping and allowed for high-quality sperm data measurement. The ovarian fluid was filtered from eggs with a fine-mesh aquarium net and refrigerated at 4^{o}C in a glass beaker. Ovarian fluid activating solutions were made at 33% concentration with water. Bovine serum albumin was included in the sperm activating solution at a concentration of 1:1000 to prevent sperm from adhering to the microscope slide (Beirão et al., 2014; Beirão et al., 2015).

Sperm swimming performance was recorded using a Prosilica GE680 camera attached to an inverted Leica DM IL LED microscope, with a 20× phase contrast objective. Approximately one µl of diluted semen was put on the edge of the chamber of a Cytonix 2 chambered slide, which had been cooled to approximately 9°C with a custom Physitemp TS-4 system. The semen was then flushed into the chamber by 395 microliters of the sperm activating solution (the test treatment). This activated the sperm and marked the start of the video, which was taken at 80 fps. The first 6s post-activation were used to locate an area of suitable sperm density and focus the microscope. Videos were captured using Streampix software, and quality checked for sperm density, motility, and proper microscope focus before being accepted into the data pool. If a video was deemed poor quality, the entire sperm activation process was repeated until three adequate videos were attained (see above). Data from these three repeated videos were averaged before analyses.

Data analyses

Sperm swimming performance comparisons were analyzed from 6.0 to 20.0s post-activation, using the Computer Assisted Sperm Analysis (CASA) plugin in ImageJ with a tracking interval of 0.5s (Purchase & Earle, 2012). Two sperm swimming performance traits were used in analyses, as these have been shown to be related to paternity under sperm competition (Alonzo et al., 2016; Evans et al., 2013, Gage et al., 2004; Lehnert et al., 2017; Young et al., 2013); the percent of the sperm cells within an ejaculate that are motile, and of the motile cells, their curvilinear swimming velocity or VCL. How ovarian fluid modified these parameters in different species of sperm – controlling for individual variation in sperm quality using a water standard, was our metric for determining conspecific sperm preference and thus the ability of females to exert cryptic female choice.

To test hypothesis (1) that ovarian fluid upregulated sperm motility and velocity regardless of species, we constructed normally distributed mixed effects generalized linear models for motility and VCL (Equation 1). Both models used the fixed effect of sperm activating solution (water or ovarian fluid – the average of all types) and the random effect of male ID as the independent variables. To simplify analyses, we elected to focus this comparison to the earliest sperm post-activation time period available (6.0-6.5 seconds) as fertilizations happen quickly (Beirão et al., 2019; Hoysak & Liley, 2001; Rosengrave et al., 2016), and thus represents the most important time interval for females to modify.

$$\%\, mot, VCL \;=\; \beta_0 + \beta_{Sperm\ Activating\ Solution} \;+\; \beta_{Male\ ID} \;+\; \varepsilon{:}\, normal$$

Equation 1

To evaluate hypothesis (2) that ovarian fluid upregulated conspecific sperm more than heterospecific sperm; we adopted a similar approach using normally distributed error, the same as equation 1. However, we used standardized swimming performance (the ratio in ovarian fluid to water) as the dependent variable, ovarian fluid type (conspecific or heterospecific) as a fixed independent variable, and Male ID as a random independent variable (Equation 2).

$$\%\, mot\ ratio, VCL\ ratio\ = \beta_0 + \beta_{Ovarian\ Fluid\ Type} + \beta_{Male\ ID} + \varepsilon: normal$$

Equation 2

To test hypothesis (3) that there were patterns of upregulation among our three species, we constructed a generalized mixed-effects model and used standardized motility and velocity as dependent variables and sperm species, ovarian fluid species (char, salmon, or trout), and their interaction as fixed independent variables, and Male ID as a random independent variable (Equation 3). Errors were normally distributed. If the interaction was significant, the model was broken down by ovarian fluid species and analyzed post-hoc to determine significant differences between the male species in each ovarian fluid.

$$\%\, mot\ ratio, VCL\ ratio\ = \beta_0 + \beta_{Sperm\ Species} + \beta_{Ovarian\ Fluid\ Species} + \beta_{Sperm\ Species * Ovarian\ Fluid\ Species} + \beta_{Male\ ID} + \varepsilon: normal$$

Equation 3

Results

Sperm of all three species had similar swimming characteristics, which declined rapidly post-activation (Figure 2-2). A declining function was expected, so we simplified subsequent results and focused on the most biologically relevant time for sperm competition; the earliest

we could capture, six seconds. Our first hypothesis that ovarian fluid upregulates sperm swimming performance compared to water in any species of sperm was supported (Figure 2-3). Motility (df=1, f=6.15, p=0.018) and curvilinear velocity (Df=,1 F=83.67, p=<0.001) were both upregulated at six seconds by 53 and 30 percent, respectively. Individual male performance was visualized following Purchase et al. (2010) to show this upregulation between water and all ovarian fluids combined (Figure 2-3). The average standardized ratios of upregulation across all three ovarian fluids were 1.5 for motility and 1.3 for velocity. Using this approach, we were then able to present the ratios of upregulation separately for each species of sperm and ovarian fluid.

Our second and third hypotheses, that ovarian fluid enables conspecific sperm preference and that there is a clear and consistent ranking in how it influences sperm of different species, were not supported. There was variation in how much ovarian fluid upregulated sperm by species, but trends were not consistent at six seconds within replicates (Appendix 2-A) or throughout full recorded time across replicates (Appendix 2-B). At 6 seconds post-activation (Figure 2-4), there was no statistically significant difference in the degree of sperm upregulation by ovarian fluid for conspecific vs. heterospecific sperm motility (df=1, F=2.25, p=0.138) and velocity (df=1, F=2.173, p=0.149). Trout ovarian fluid did upregulate trout sperm the most, but char and salmon ovarian fluid upregulated trout sperm more than sperm of their own species (Figure 2-4). The interaction between male and female species was not significant for motility (df=4, F=0.61, p=0.66), but was for velocity (df=4, F=3.8841, p=0.007). When the interaction was broken down and analyzed separately for each ovarian fluid species,

the only significant difference in velocity at six seconds was between char and trout sperm in salmon ovarian fluid (df=33, t=-3.513, p=0.004).

Discussion

Conspecific sperm preference can be a crucial mechanism in preventing hybridization and has been demonstrated in taxa as diverse as mussels (Klibansky & McCartney, 2014), crickets (Howard et al., 1998; Tyler et al., 2013), birds (Pizzari & Birkhead, 2000; Wagner et al., 2004), and European populations in two of our study species (Yeates et al., 2013). We, therefore, expected to find that ovarian fluid mediated upregulation of sperm swimming [predicted to enable conspecific sperm preference] would be strong in our study system of hybridizing salmonids but found that while ovarian fluid as a whole consistently increased sperm swimming performance when compared to water, it did not do so when examined in terms of conspecific sperm vs. heterospecific sperm. This implies that conspecific sperm preference is not a significant defense against hybridization and consequent ecological waste of gametes or introgression of hybrid genes in our specific study populations within this model system. More research, however, is needed to confirm this, preferably over a range of systems where these fish have interacted for differing amounts of time. Given that some of our study populations were completely allopatric, this could have led to our lack of results when compared among species. However, since there was not strong evidence for conspecific sperm preference in our study population of brown trout that had been exposed to brook char, this may not be case. Examining conspecific sperm preference in other populations would allow us to determine if a) conspecific sperm preference varies among populations in Newfoundland,

and b) prolonged history of interaction is required to produce a strong conspecific sperm preference.

Ovarian fluid did have a clear effect on sperm swimming performance. When compared to water, ovarian fluid upregulated sperm motility and velocity throughout the swimming lifespan of the sperm cells. This is no surprise because the components of ovarian fluid (Lehnert et al., 2017; Rosengrave et al., 2009) have been shown to prolong sperm lifespan and increase sperm velocity in these and related taxa (Elofsson et al., 2006; Evans et al., 2013; Urbach et al., 2005). Since this function of ovarian fluid was strongly demonstrated, we predicted to see ovarian fluid mediated conspecific sperm preference following other taxa (Klibansky & McCartney, 2014) and previous work with two of our three species (Yeates et al., 2013). It is important to note that while we examined a predictor of conspecific sperm preference further studies examining sperm competition would be required to confirm the presence or absence of conspecific sperm preference in this study system.

We did not find evidence of conspecific upregulation of sperm behaviour in our Newfoundland populations of salmonid fishes. Ovarian fluid did not upregulate conspecific sperm more than heterospecific sperm. At the species level, trout sperm was upregulated more in trout ovarian fluid, but trout sperm was also upregulated more than the others in the two heterospecific ovarian fluids. This result is surprising because of the high energetic cost to females linked to mating with the wrong species of male. Atlantic salmon and brook char do not create viable adult hybrids (Chevassus, 1979), brown trout and brook char create sterile adults – known as tiger trout (Buss & Wright, 1958), and Atlantic salmon and brown trout create sterile F2s (Chevassus, 1979). In all these cases, hybrid fertilizations of a female's eggs create

evolutionary dead ends. The absence of conspecific sperm preference in our system creates problems due to the prevalence of sneaker males (Weir et al., 2016), which can circumvent female mate choice and steal fertilizations, which can increase rates of hybridization (Garcia-Vazquez et al., 2002). The prevalence of sneak spawning in Newfoundland Atlantic salmon (Hutchings, 1985), as well as the potential for hybridization across three species, makes this system ideal for studying cryptic female choice.

This is the first investigation of conspecific sperm preference in the context of hybridization for brook char. Yeates et al. (2013) examined hybridization with European populations of native Atlantic salmon and brown trout. They found that 1) ovarian fluid was strongly linked to conspecific sperm preference and 2) that the egg itself did not have any protections against hybridization. Given that Yeates et al. (2013) used naturally sympatric populations of Atlantic salmon and brown trout, it follows that strong conspecific sperm preference would have evolved over time to minimize hybridizations. Our study system has Atlantic salmon that have been isolated from European salmon for 600,000 years (Lehnert et al., 2020), introduced and invasive brown trout, and documented wild hybridization (McGowan & Davidson, 1992), which follows what we know about other invaded salmonid systems. Salmonids are well known to hybridize with novel invaders (Poulos, 2019), and hybrids resulting from these invasions have been well-documented across the family (DeHaan et al., 2010; McGowan & Davidson, 1992; Muhlfeld et al., 2017). Given that all hybridization between our three study species results in evolutionary dead-ends (Chevassus, 1979) that impart costs to mating females, this variation from the documented strength of conspecific sperm preference (Yeates et al., 2013) strongly suggests that other mechanisms, such as behavioural isolation

through pre-fertilization mate choice for conspecific males, may be at work to lower occurrences of hybridization in our populations (Sorensen et al., 1995). To best examine cryptic female choice or other mechanisms to prevent hybridization in salmonids, a wide variety of different species and assemblages over a range of allopatric and sympatric distributions should be considered.

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Literature cited

Abbott, R., Albach, D., Ansell, S., Arntzen, J. W., Baird, S. J. E., Bierne, N., Boughman, J., Brelsford, A., Buerkle, C. A., Buggs, R., Butlin, R. K., Dieckmann, U., Eroukhmanoff, F., Grill, A., Cahan, S. H., Hermansen, J. S., Hewitt, G., Hudson, A. G., Jiggins, C., … Zinner, D. (2013). Hybridization and speciation. *Journal of Evolutionary Biology*, *26*(2), 229–246. https://doi.org/10.1111/j.1420-9101.2012.02599.x

Alonzo, S. H., Stiver, K. A., & Marsh-Rollo, S. E. (2016). Ovarian fluid allows directional cryptic female choice despite external fertilization. *Nature Communications*, *7*(1), 12452. https://doi.org/10.1038/ncomms12452

Andersson, M. (1994). *Sexual Selection* (2nd ed.). https://press.princeton.edu/books/paperback/9780691000572/sexual-selection

Bateman, A. J. (1948). Intra-sexual selection in *Drosophila*. *Heredity*, *2*(3), 349–368. https://doi.org/10.1038/hdy.1948.21

Beirão, J., Purchase, C. F., Wringe, B. F., & Fleming, I. A. (2015). Inter-population ovarian fluid variation differentially modulates sperm motility in Atlantic cod (*Gadus morhua)*: *Gadus morhua* ovarian fluid affects sperm motility. *Journal of Fish Biology*, *87*(1), 54–68. https://doi.org/10.1111/jfb.12685

Beirão, J, Purchase, C., Wringe, B., & Fleming, I. (2014). Sperm plasticity to seawater temperatures in Atlantic cod (*Gadus morhua)* is affected more by population origin than individual environmental exposure. *Marine Ecology Progress Series*, *495*, 263–274. https://doi.org/10.3354/meps10552

Beirão, J, Egeland, T. B., Purchase, C. F., & Nordeide, J. T. (2019). Fish sperm competition in hatcheries and between wild and hatchery origin fish in nature. *Theriogenology*, *133*, 201–209. https://doi.org/10.1016/j.theriogenology.2019.03.034

Buss, K., & Wright, J. E. (1958). Appearance and fertility of trout hybrids. *Transactions of the American Fisheries Society*, *87*(1), 172–181. https://doi.org/10.1577/1548-8659(1957)87[172: AAFOTH]2.0.CO;2

Butts, I. A. E., Johnson, K., Wilson, C. C., & Pitcher, T. E. (2012). Ovarian fluid enhances sperm velocity based on relatedness in lake trout, (*Salvelinus namaycush)*. *Theriogenology*, *78*(9), 2105-2109.e1. https://doi.org/10.1016/j.theriogenology.2012.06.031

Castillo, D. M., & Moyle, L. C. (2019). Conspecific sperm precedence is reinforced, but postcopulatory sexual selection weakened, in sympatric populations of *Drosophila*. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1899), 20182535. https://doi.org/10.1098/rspb.2018.2535

Chevassus, B. (1979). Hybridization in salmonids: Results and perspectives. *Aquaculture*, *17*(2), 113–128. https://doi.org/10.1016/0044-8486(79)90047-4

Close, R. L., & Bell, J. N. (1997). Fertile hybrids in two genera of wallabies: *Petrogale* and *Thylogale*. *Journal of Heredity*, *88*(5), 393–397. https://doi.org/10.1093/oxfordjournals.jhered.a023124

Dean, R., Nakagawa, S., Pizzari, T., Svensson, A. E. E., & Shaw, E. R. G. (2011). The risk and intensity of sperm ejection in female birds. *The American Naturalist*, *178*(3), 343–354. https://doi.org/10.1086/661244

DeHaan, P. W., Schwabe, L. T., & Ardren, W. R. (2010). Spatial patterns of hybridization between bull trout, (*Salvelinus confluentus)*, and brook trout, (*Salvelinus fontinalis*) in an Oregon stream network. *Conservation Genetics*, *11*(3), 935–949. https://doi.org/10.1007/s10592-009-9937-6

Dietrich, G. J., Wojtczak, M., Słowińska, M., Dobosz, S., Kuźmiński, H., & Ciereszko, A. (2008). Effects of ovarian fluid on motility characteristics of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. *Journal of Applied Ichthyology*, *24*(4), 503–507. https://doi.org/10.1111/j.1439-0426.2006.01130.x

Dixson, A. (2003). Sexual selection by cryptic female choice and the evolution of primate sexuality. *Evolutionary Anthropology: Issues, News, and Reviews*, *11*(S1), 195–199. https://doi.org/10.1002/evan.10090

Eberhard, W. G. (2010). Evolution of genitalia: Theories, evidence, and new directions. *Genetica*, *138*(1), 5–18. https://doi.org/10.1007/s10709-009-9358-y

Elofsson, H., Look, K. J. W. V., Sundell, K., Sundh, H., & Borg, B. (2006). Stickleback sperm saved by salt in ovarian fluid. *Journal of Experimental Biology*, *209*(21), 4230–4237. https://doi.org/10.1242/jeb.02481

Emery Thompson, M., & Georgiev, A. V. (2014). The high price of success: Costs of mating effort in male primates. *International Journal of Primatology*, *35*(3), 609–627. https://doi.org/10.1007/s10764-014-9790-4

Esteve, M. (2005). Observations of spawning behaviour in Salmoninae: *Salmo, Oncorhynchus* and *Salvelinus*. *Reviews in Fish Biology and Fisheries*, *15*, 1–21. https://doi.org/10.1007/s11160-005-7434-7Evans, J. P., Rosengrave, P., Gasparini, C., &

Gemmell, N. J. (2013). Delineating the roles of males and females in sperm competition. *Proceedings of the Royal Society B: Biological Sciences*, *280*(1772). https://doi.org/10.1098/rspb.2013.2047

Ferkin, M. H. (2018). Odor Communication and Mate Choice in Rodents. *Biology (Basel)*, 7*(1), 13*. https://doi.org/10.3390/biology7010013

Firman, R. C. (2011). Polyandrous females benefit by producing sons that achieve high reproductive success in a competitive environment. *Proceedings of the Royal Society B: Biological Sciences*, *278*(1719), 2823–2831. https://doi.org/10.1098/rspb.2010.2791

Firman, R. C., Gasparini, C., Manier, M. K., & Pizzari, T. (2017). Postmating female control: 20 years of cryptic female choice. *Trends in Ecology & Evolution*, *32*(5), 368–382. https://doi.org/10.1016/j.tree.2017.02.010

Gage, M. J. G., Macfarlane, C. P., Yeates, S., Ward, R. G., Searle, J. B., & Parker, G. A. (2004). Spermatozoal traits and sperm competition in Atlantic salmon: Relative sperm velocity Is the primary determinant of fertilization success. *Current Biology*, *14*(1), 44–47. https://doi.org/10.1016/j.cub.2003.12.028

Galvano, P. M., Johnson, K., Wilson, C. C., Pitcher, T. E., & Butts, I. A. E. (2013). Ovarian fluid influences sperm performance in lake trout, (*Salvelinus namaycush*). *Reproductive Biology*, *13*(2), 172–175. https://doi.org/10.1016/j.repbio.2013.02.001

Garcia-Vazquez, E., Moran, P., Perez, J., Martinez, J. L., Izquierdo, J. I., de Gaudemar, B., & Beall, E. (2002). Interspecific barriers between salmonids when hybridisation is due to sneak mating. *Heredity*, *89*(4), 288–292. https://doi.org/10.1038/sj.hdy.6800134

Garner, S. R., & Neff, B. D. (2013). Alternative male reproductive tactics drive asymmetrical hybridization between sunfishes (*Lepomis spp.*). *Biology Letters*, *9*(6), 20130658. https://doi.org/10.1098/rsbl.2013.0658

Gross, M. R. (1996). Alternative reproductive strategies and tactics: Diversity within sexes. *Trends in Ecology & Evolution, 11*(2), 92-98. https://doi.org/10.1016/0169-5347(96)81050-0

Haddeland, P. J., Junge, C., Serbezov, D., & Vøllestad, L. A. (2015). Genetic Parentage Analysis Confirms a Polygynandrous Breeding System in the European Grayling (Thymallus thymallus). *PLOS ONE*, *10*(3), e0122032. https://doi.org/10.1371/journal.pone.0122032

Holcomb, M., Woolsey, J., Cloud, J. G., & Ingermann, R. L. (2004). Effects of Clove Oil, Tricaine, and CO2 on Gamete Quality in Steelhead and White Sturgeon. *North American Journal of Aquaculture*, *66*(3), 228–233. https://doi.org/10.1577/A03-027.1

Howard, D. J., Gregory, P. G., Chu, J., & Cain, M. L. (1998). Conspecific sperm precedence is an effective barrier to hybridization between closely related species. *Evolution*, *52*(2), 511–516. https://doi.org/10.2307/2411086

Hoysak, D. J., & Liley, N. R. (2001). Fertilization dynamics in sockeye salmon and a comparison of sperm from alternative male phenotypes. *Journal of Fish Biology*, *58*(5), 1286–1300. https://doi.org/10.1111/j.1095-8649.2001.tb02286.x

Hustins, D. (2007). Brown trout and rainbow trout: A journey into Newfoundland waters. Tight Lines Publishers.

Hutchings, J. A. (1985). *The adaptive significance of lakeward migrations by juvenile Atlantic salmon, Salmo salar L.* [Masters, Memorial University of Newfoundland]. https://research.library.mun.ca/4104/

Jennions, M. D., & Petrie, M. (1997). Variation in mate choice and mating preferences: A review of causes and consequences. *Biological Reviews*, *72*(2), 283–327. https://doi.org/10.1111/j.1469-185X.1997.tb00015.x

Jones, A. G., & Ratterman, N. L. (2009). Mate choice and sexual selection: What have we learned since Darwin? *Proceedings of the National Academy of Sciences*, *106*(Supplement 1), 10001–10008. https://doi.org/10.1073/pnas.0901129106

Klibansky, L. K. J., & McCartney, M. A. (2014). Conspecific sperm precedence is a reproductive barrier between free-spawning marine mussels in the northwest Atlantic *Mytilus* Hybrid Zone. *PLoS ONE*, *9*(9), e108433. https://doi.org/10.1371/journal.pone.0108433

Kuijper, B., Pen, I., & Weissing, F. J. (2012). A guide to sexual selection theory. *Annual Review of Ecology, Evolution, and Systematics*, *43*(1), 287–311. https://doi.org/10.1146/annurev-ecolsys-110411-160245

Lahnsteiner, F. (2002). The influence of ovarian fluid on the gamete physiology in the Salmonidae. *Fish Physiology and Biochemistry*, *27*(1), 49–59. https://doi.org/10.1023/B:FISH.0000021792.97913.2e

Landry, C., Garant, D., Duchesne, P., & Bernatchez, L. (2001). 'Good genes as heterozygosity': The major histocompatibility complex and mate choice in Atlantic salmon (*Salmo salar*). *Proceedings of the Royal Society B: Biological Sciences*, *268*(1473), 1279–1285. https://doi.org/10.1098/rspb.2001.1659

Lehnert, S. J., Butts, I. a. E., Flannery, E. W., Peters, K. M., Heath, D. D., & Pitcher, T. E. (2017). Effects of ovarian fluid and genetic differences on sperm performance and fertilization success of alternative reproductive tactics in chinook salmon. *Journal of Evolutionary Biology*, *30*(6), 1236–1245. https://doi.org/10.1111/jeb.13088

Lehnert, Sarah J., Kess, T., Bentzen, P., Clément, M., & Bradbury, I. R. (2020). Divergent and linked selection shape patterns of genomic differentiation between European and North American Atlantic salmon (*Salmo salar*). *Molecular Ecology*, *29*(12), 2160–2175. https://doi.org/10.1111/mec.15480

Lymbery, R. A., Kennington, W. J., & Evans, J. P. (2017). Egg chemoattractants moderate intraspecific sperm competition. *Evolution Letters*, *1*(6), 317–327. https://doi.org/10.1002/evl3.34

McGowan, C., & Davidson, W. S. (1992). Unidirectional natural hybridization between brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*) in Newfoundland. *Canadian Journal of Fisheries and Aquatic Sciences*, *49*(9), 1953–1958. https://doi.org/10.1139/f92-216

Morina, D. L., Demarais, S., Strickland, B. K., & Larson, J. E. (2018). While males fight, females choose: Male phenotypic quality informs female mate choice in mammals. *Animal Behaviour*, *138*, 69–74. https://doi.org/10.1016/j.anbehav.2018.02.004

Muhlfeld, C. C., Kovach, R. P., Al-Chokhachy, R., Amish, S. J., Kershner, J. L., Leary, R. F., Lowe, W. H., Luikart, G., Matson, P., Schmetterling, D. A., Shepard, B. B., Westley, P. A. H., Whited, D., Whiteley, A., & Allendorf, F. W. (2017). Legacy introductions and climatic

variation explain spatiotemporal patterns of invasive hybridization in a native trout. *Global Change Biology*, *23*(11), 4663–4674. https://doi.org/10.1111/gcb.13681

Parker, G. A. (1970). Sperm competition and its evolutionary consequences in the insects. *Biological Reviews*, *45*(4), 525–567. https://doi.org/10.1111/j.1469-185X.1970.tb01176.x

Pizzari, T., & Birkhead, T. R. (2000). Female feral fowl eject sperm of subdominant males. *Nature*, *405*(6788), 787–789. https://doi.org/10.1038/35015558

Poulos, S. (2019). Embryo development under context of hybridization and a review of regional patterns in hybrid frequency of Atlantic salmon and brown trout [Masters, Memorial University of Newfoundland]. https://research.library.mun.ca/13969/

Purchase, C. F., & Moreau, D. T. R. (2012). Stressful environments induce novel phenotypic variation: Hierarchical reaction norms for sperm performance of a pervasive invader. *Ecology and Evolution*, *2*(10), 2567–2576. https://doi.org/10.1002/ece3.364

Purchase, C. F., & Rooke, A. C. (2020). Freezing ovarian fluid does not alter how it affects fish sperm swimming performance: Creating a cryptic female choice 'spice rack' for use in split-ejaculate experimentation. *Journal of Fish Biology*, *96*(3), 693–699. https://doi.org/10.1111/jfb.14263

Purchase, C. F., & Earle, P. T. (2012). Modifications to the IMAGEJ computer assisted sperm analysis plugin greatly improve efficiency and fundamentally alter the scope of attainable data. *Journal of Applied Ichthyology*, *28*(6), 1013–1016. https://doi.org/10.1111/jai.12070

Remick, D. (1992). Measuring the costs of reproduction. *Trends in Ecology & Evolution*, *7*(2), 42–45. https://doi.org/10.1016/0169-5347(92)90104-J

Rooke, A. C., Palm-Flawd, B., & Purchase, C. F. (2020). The impact of a changing winter climate on the hatch phenology of one of North America's largest Atlantic salmon populations. *Conservation Physiology*, *7*(1). https://doi.org/10.1093/conphys/coz015

Rosengrave, P., Montgomerie, R., & Gemmell, N. (2016). Cryptic female choice enhances fertilization success and embryo survival in chinook salmon. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1827), 20160001. https://doi.org/10.1098/rspb.2016.0001

Rosengrave, P., Taylor, H., Montgomerie, R., Metcalf, V., McBride, K., & Gemmell, N. J. (2009). Chemical composition of seminal and ovarian fluids of chinook salmon (*Oncorhynchus tshawytscha*) and their effects on sperm motility traits. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, *152*(1), 123–129. https://doi.org/10.1016/j.cbpa.2008.09.009

Sorensen, P. W., Essington, T., Weigel, D. E., & Cardwell, J. R. (1995). Reproductive interactions between sympatric brook and brown trout in a small Minnesota stream. *Canadian Journal of Fisheries and Aquatic Sciences*, *52*(9), 1958-1965.https://doi.org/10.1139/f95-787

Trivers, R. L. (1972). Sexual election and the Descent of Man*, 1871-1971*. Aldine, Chicago.

Tyler, F., Harrison, X. A., Bretman, A., Veen, T., Rodríguez-Muñoz, R., & Tregenza, T. (2013). Multiple post-mating barriers to hybridization in field crickets. *Molecular Ecology*, *22*(6), 1640–1649. https://doi.org/10.1111/mec.12187

Tynkkynen, K., Raatikainen, K. J., Häkkilä, M., Haukilehto, E., & Kotiaho, J. S. (2009). Alternative reproductive tactics and the propensity of hybridization. *Journal of Evolutionary Biology*, *22*(12), 2512–2518. https://doi.org/10.1111/j.1420-9101.2009.01870.x

Urbach, D., Folstad, I., & Rudolfsen, G. (2005). Effects of ovarian fluid on sperm velocity in Arctic charr (*Salvelinus alpinus*). *Behavioral Ecology and Sociobiology*, *57*(5), 438–444. https://doi.org/10.1007/s00265-004-0876-4

Wagner, R. H., Helfenstein, F., & Danchin, E. (2004). Female choice of young sperm in a genetically monogamous bird. *Proceedings of the Royal Society B: Biological Sciences*, *271*(Suppl 4), S134–S137.

Weir, L. K., Kindsvater, H. K., Young, K. A., & Reynolds, J. D. (2016). Sneaker males affect fighter male body size and sexual size dimorphism in salmon. *The American Naturalist*, *188*(2), 264–271. https://doi.org/10.1086/687253

Westley, P. A. H., & Fleming, I. A. (2011). Landscape factors that shape a slow and persistent aquatic invasion: Brown trout in Newfoundland 1883–2010. *Diversity and Distributions*, *17*(3), 566–579. https://doi.org/10.1111/j.1472-4642.2011.00751.x

Wilson, A. C., Maxson, L. R., & Sarich, V. M. (1974). Two types of molecular evolution. Evidence from studies of interspecific hybridization. *Proceedings of the National Academy of Sciences*, *71*(7), 2843–2847. https://doi.org/10.1073/pnas.71.7.2843

Yeates, S. E., Diamond, S. E., Einum, S., Emerson, B. C., Holt, W. V., & Gage, M. J. G. (2013). Cryptic choice of conspecific sperm controlled by the impact of ovarian fluid on sperm swimming behavior. *Evolution*, *67*(12), 3523–3536. https://doi.org/10.1111/evo.12208

Yeates, S. E., Einum, S., Fleming, I. A., Megens, H.-J., Stet, R. J. M., Hindar, K., Holt, W. V., Van Look, K. J. W., & Gage, M. J. G. (2009). Atlantic salmon eggs favour sperm in competition that have similar major histocompatibility alleles. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1656), 559–566. https://doi.org/10.1098/rspb.2008.1257

Young, B., Conti, D. V., & Dean, M. D. (2013). Sneaker "jack" males outcompete dominant "hooknose" males under sperm competition in chinook salmon (*Oncorhynchus tshawytscha*). *Ecology and Evolution*, *3*(15), 4987–499. https://doi.org/10.1002/ece3.869

Chapter 2 tables

Table 2-1: Diagram of one experimental replicate. We repeated each of these replicates procedurally three times. Legend: AS= Atlantic salmon, BC=Brook Char, BT= Brown Trout, *=congeneric, **=heterogeneric.

Replicate	Ovarian Fluid Species	Female ID	Treatment	Sperm species	Male ID	Comparison (F x M)
1	Salmon	AS1	Conspecific	Salmon	AS1	AS1xAS1
1	Salmon	AS1	Heterospecific**	Char	BC1	AS1xBC1
1	Salmon	AS1	Heterospecific*	Trout	BT1	AS1xBT1
1	Char	BC1	Conspecific	Char	BC1	BC1xBC1
1	Char	BC1	Heterospecific**	Salmon	AS1	BC1xAS1
1	Char	BC1	Heterospecific**	Trout	BT1	BC1xBT1
1	Trout	BT1	Conspecific	Trout	BT1	BT1xBT1
1	Trout	BT1	Heterospecific**	Char	BC1	BT1xBC1
1	Trout	BT1	Heterospecific*	Salmon	AS1	BT1xAS1
1	Water	W	Control	Trout	BT1	WxBT1
1	Water	W	Control	Char	BC1	WxBC1
1	Water	W	Control	Salmon	AS1	WxAS1

Chapter 2 figures

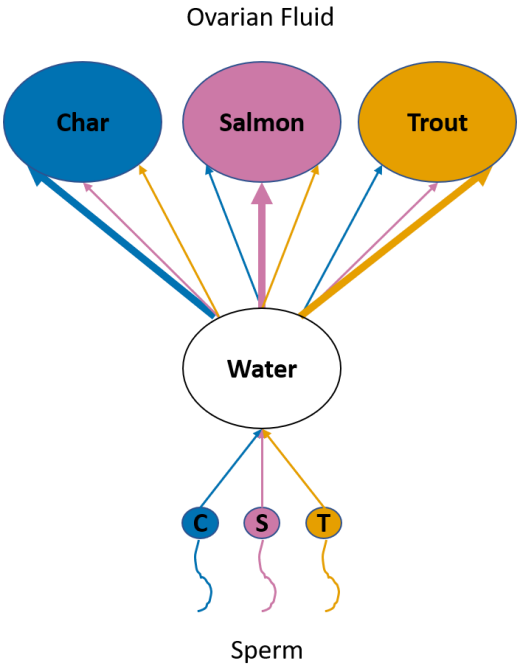


Figure 2-1: Schematic of the conceptual design. Small circles with tails are sperm, while large circles represent ovarian fluid and water. Arrows represent sperm velocity in water or ovarian fluid (the same semen sample was tested in both as separate aliquots of individual sperm). Ovarian fluids were predicted to show conspecific sperm preference, indicated by greater upregulation of conspecific sperm velocity (bolded arrows) over heterospecific sperm velocity (un-bolded arrows). Upregulation was quantified as the ratio of sperm swimming performance in ovarian fluid compared to that in water, which controls for individual variation in male quality (variable performance among males in water).

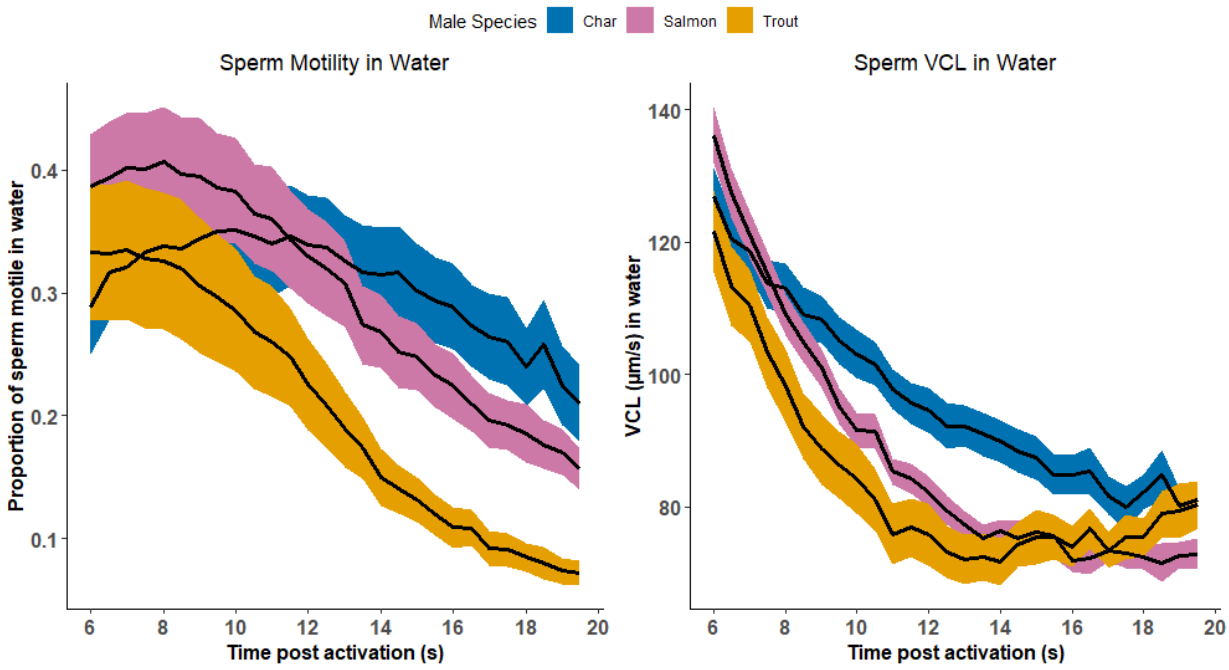


Figure 2-2: Sperm swimming performance in water (panel A: proportion motile, B: curvilinear swimming velocity [VCL]) from 6.0-20.0s post-activation. Black lines represent the average at each 0.5s interval, and colored bands are standard error among 12 individual males within a species (blue = char, pink = salmon, orange = trout).

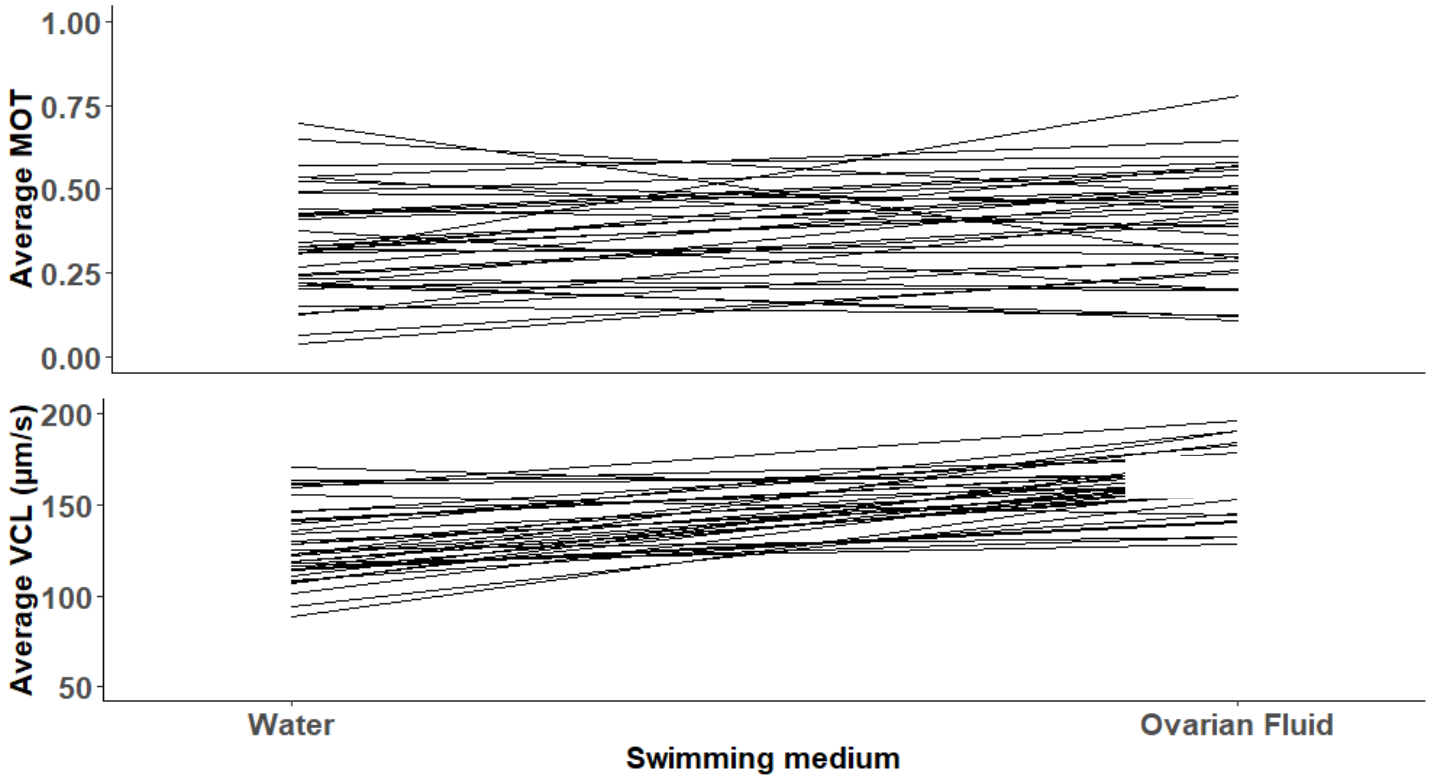


Figure 2-3: Reaction norms (top panel = proportion sperm motile, bottom = curvilinear swimming velocity) comparing sperm swimming performance from 6.0-6.5s post-activation in water to the average value in 3 ovarian fluid species. Each line represents an individual male; positive slopes indicate up-regulation of sperm swimming by ovarian fluid. Lines are created by two points, means for water represent three experimental replicates for each male, while those for ovarian fluid are from nine activations (3 experimental replications from each of 3 species of ovarian fluid).

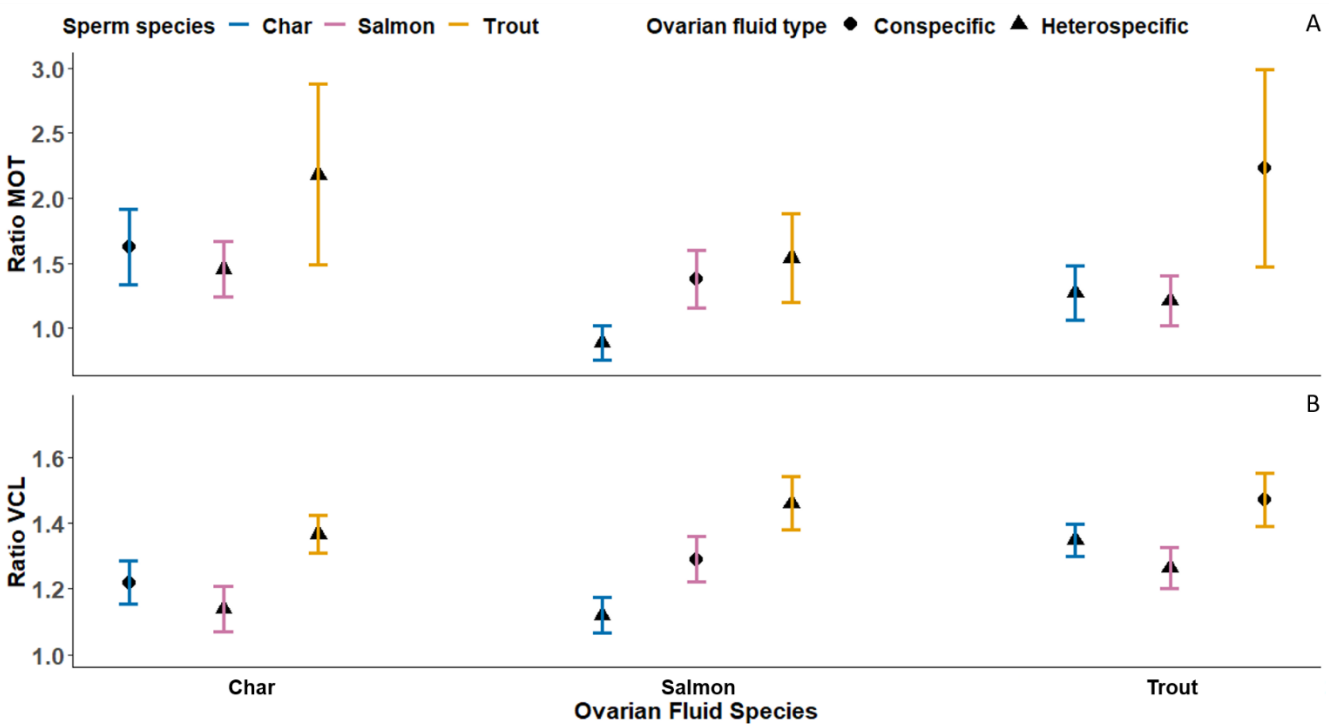
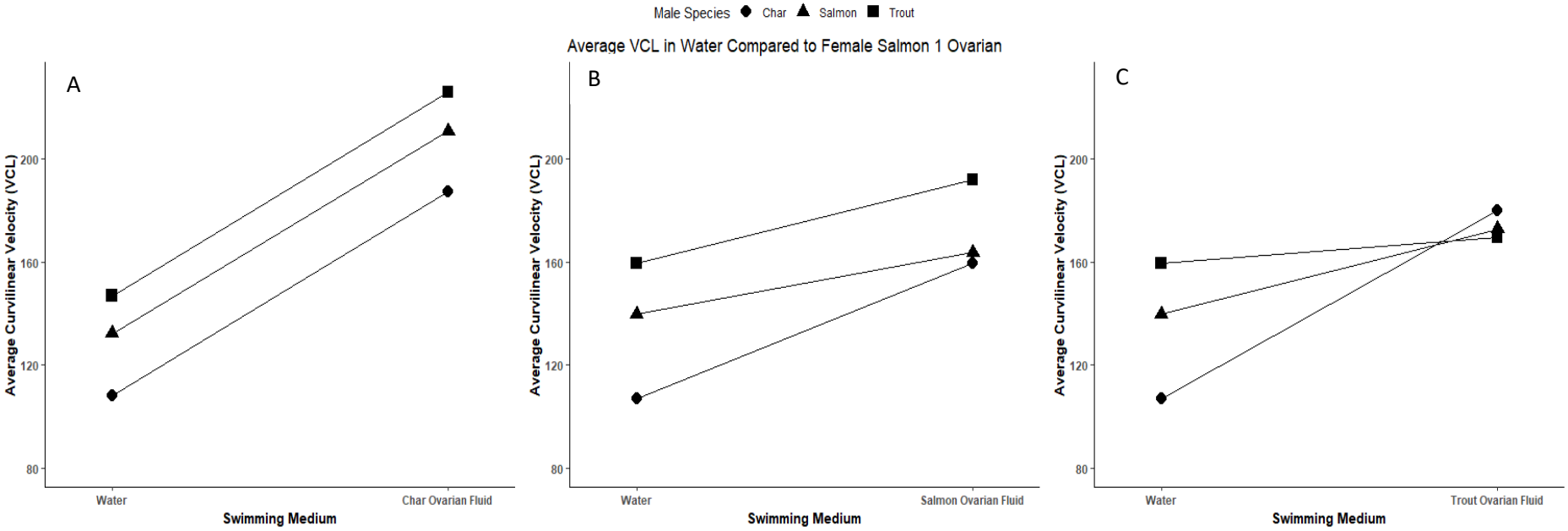


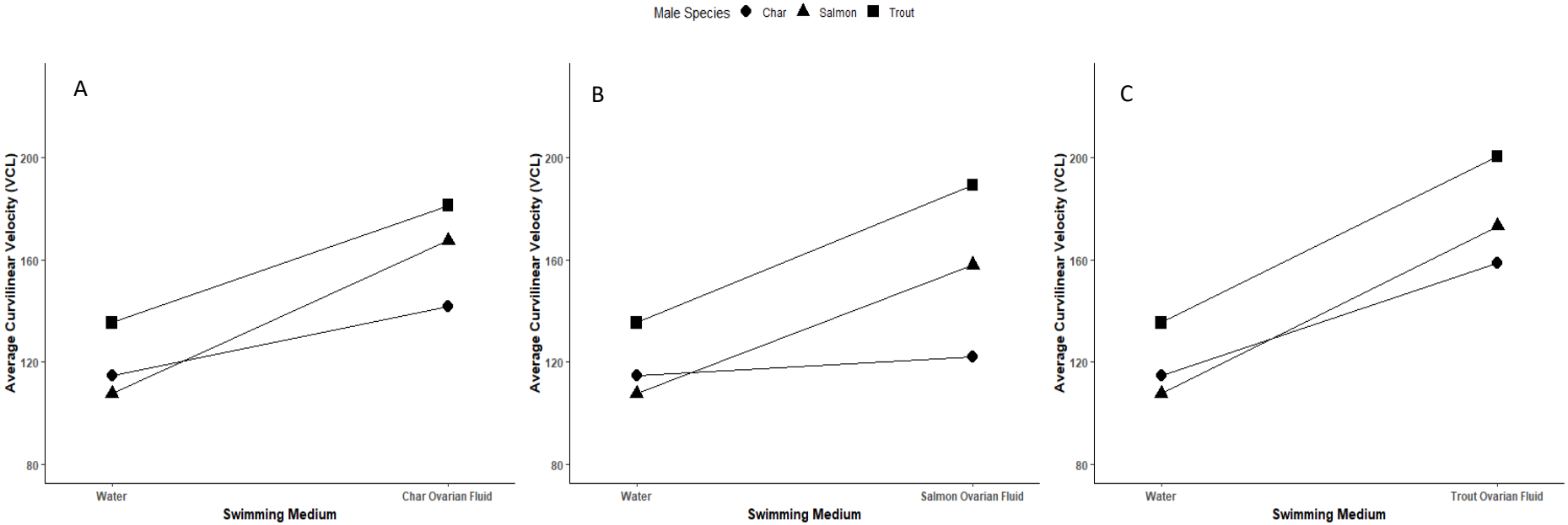
Figure 2-4: Ratio of (A) sperm motility (MOT) and (B) curvilinear velocity (VCL µm/s) in specific ovarian fluid compared to water from 6.0-6.5s post-activation – any value above 1.0 indicates upregulation in ovarian fluid. Black shapes represent the average, and colored brackets standard error among 12 males within a species. Circles represent conspecific comparisons and triangles represent heterospecific.

Chapter 2 appendices

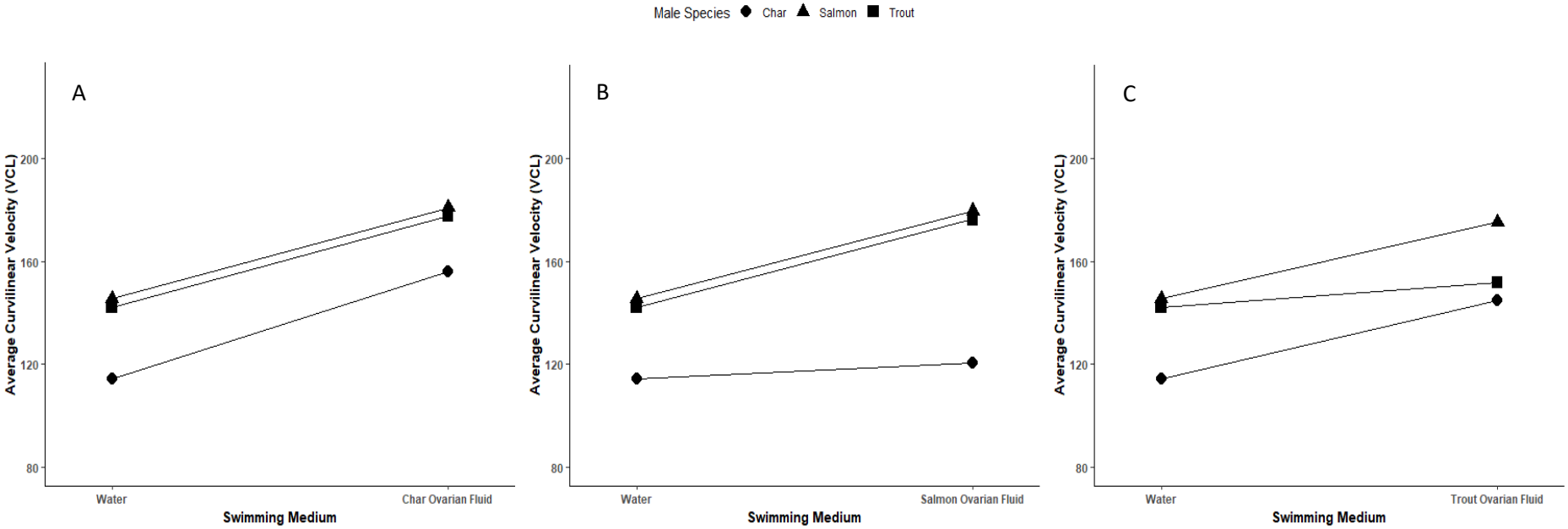
Appendix 2-A: Average VCL in water and ovarian fluid by block



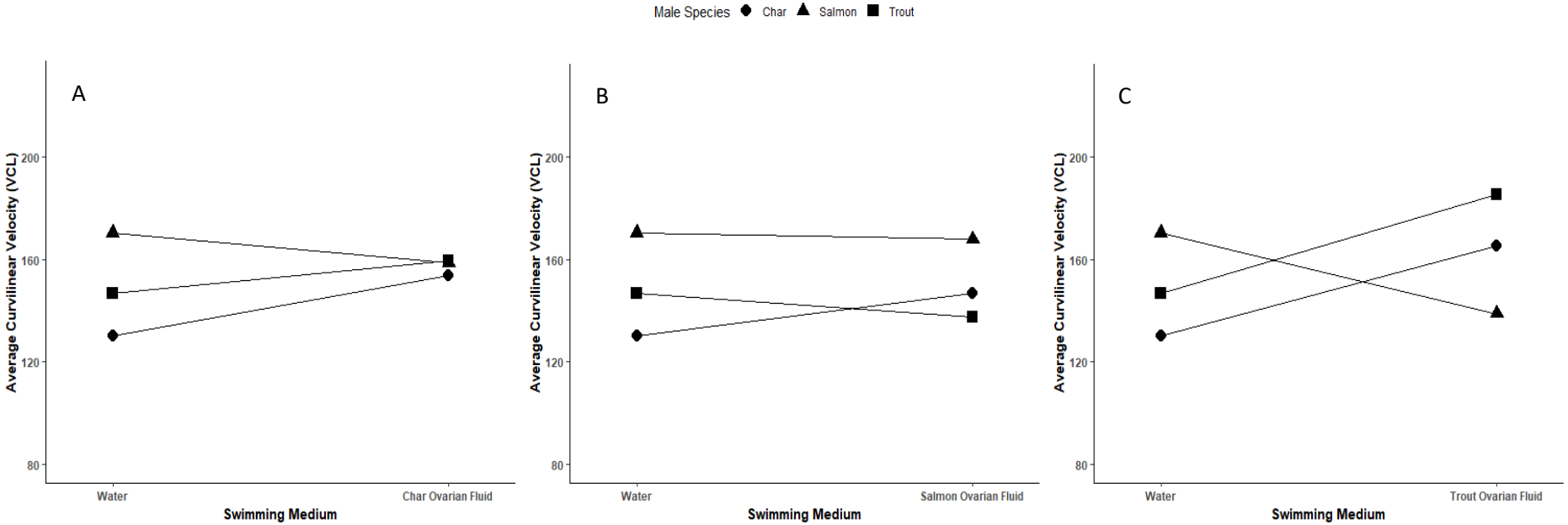
Appendix Figure 2-A1: Average VCL of sperm from replicate 1 males in water compared to ovarian fluid from the replicate 1 char (A), salmon (B), and trout (C) female. Circles represent char sperm, triangles represent salmon sperm, and squares represent trout sperm.



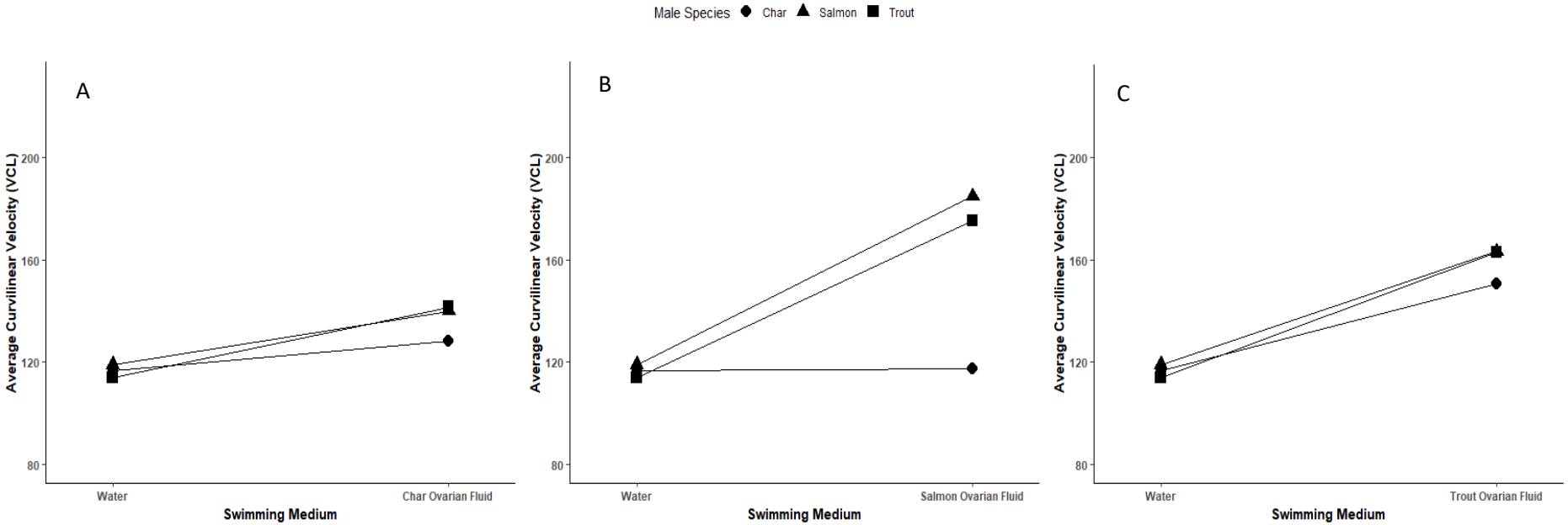
Appendix Figure 2-A2: Average VCL of sperm from replicate 2 males in water compared to ovarian fluid from the replicate 2 char (A), salmon (B), and trout (C) female. Circles represent char sperm, triangles represent salmon sperm, and squares represent trout sperm.



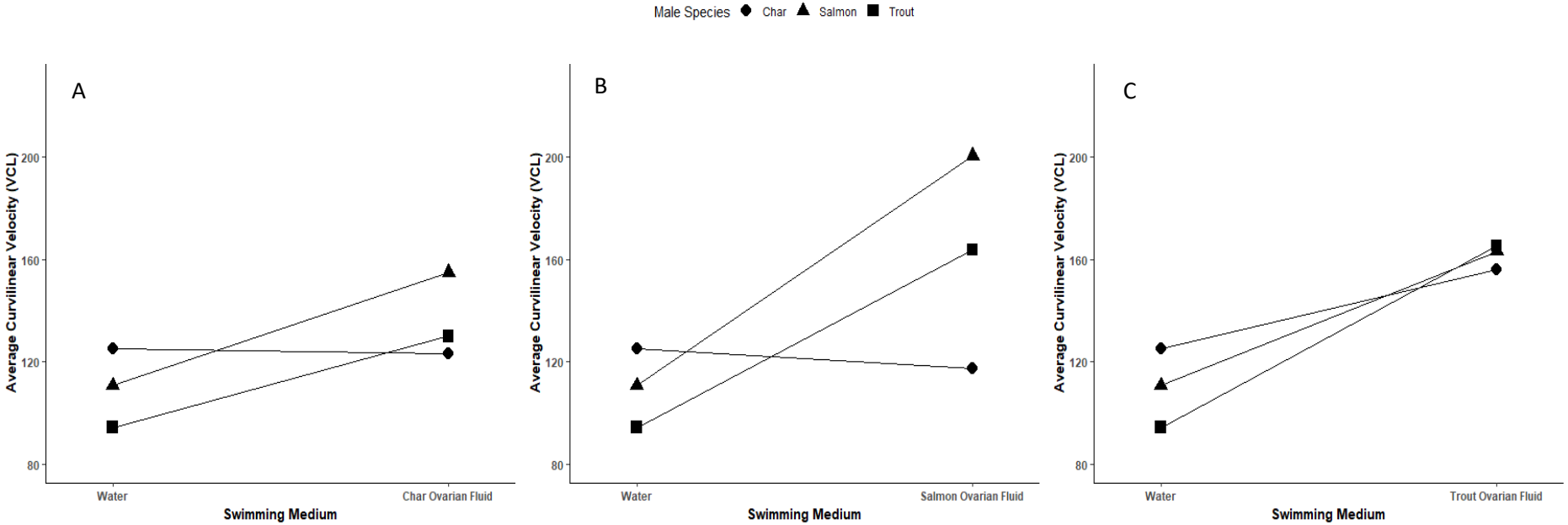
Appendix Figure 2-A3: Average VCL of sperm from replicate 3 males in water compared to ovarian fluid from the replicate 3 char (A), salmon (B), and trout (C) female. Circles represent char sperm, triangles represent salmon sperm, and squares represent trout sperm.



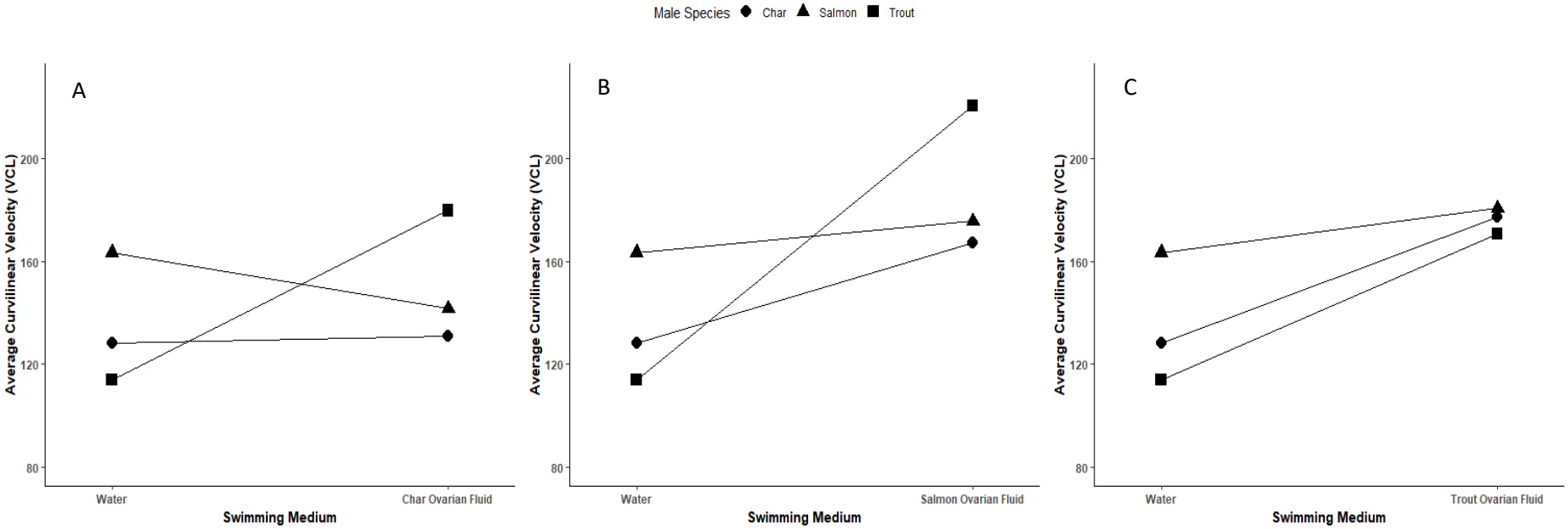
Appendix Figure 2-A4: Average VCL of sperm from replicate 4 males in water compared to ovarian fluid from the replicate 4 char (A), salmon (B), and trout (C) female. Circles represent char sperm, triangles represent salmon sperm, and squares represent trout sperm.



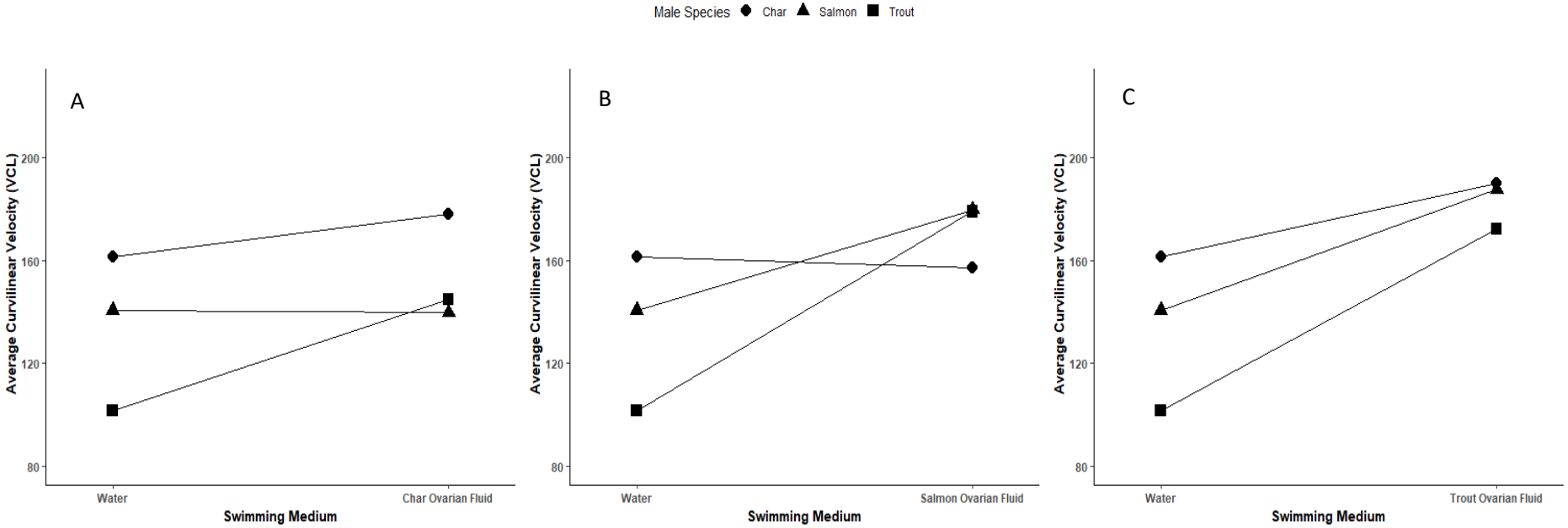
Appendix Figure 2-A5: Average VCL of sperm from replicate 5 males in water compared to ovarian fluid from the replicate 5 char (A), salmon (B), and trout (C) female. Circles represent char sperm, triangles represent salmon sperm, and squares represent trout sperm. One char and salmon male were used in replicate 6 as well.



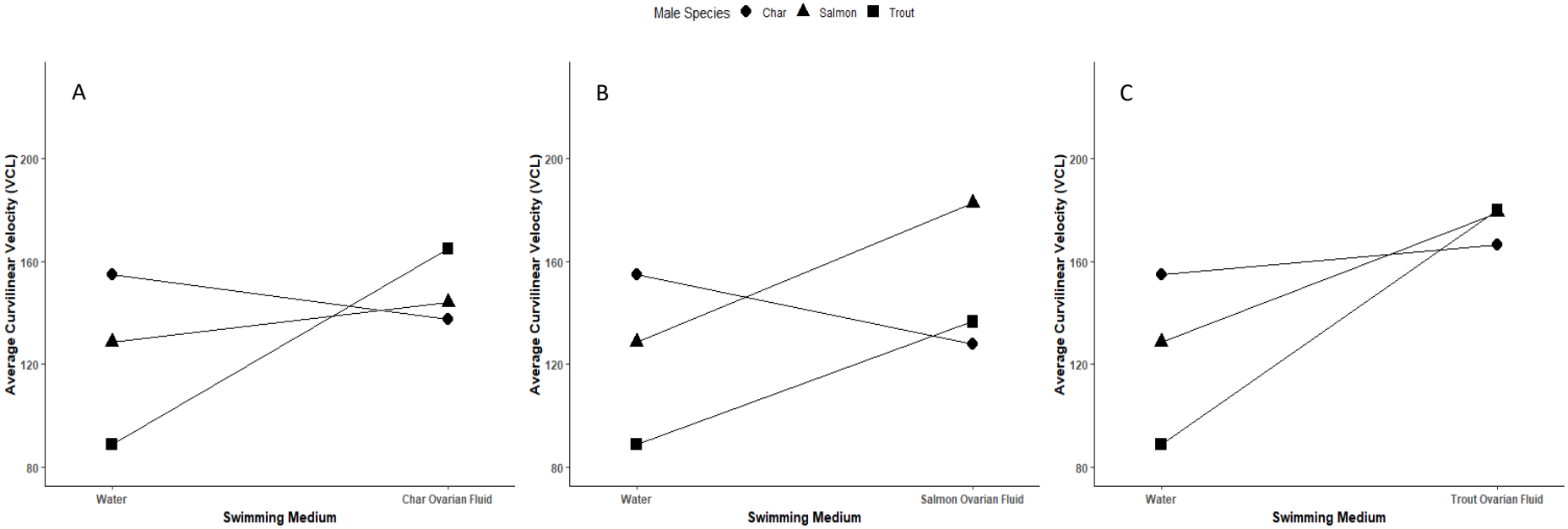
Appendix Figure 2-A6: Average VCL of sperm from replicate 6 males in water compared to ovarian fluid from the replicate 6 char (A), salmon (B), and trout (C) females. Circles represent char sperm, triangles represent salmon sperm, and squares represent trout sperm. One char and salmon male were used in replicate 5 as well.



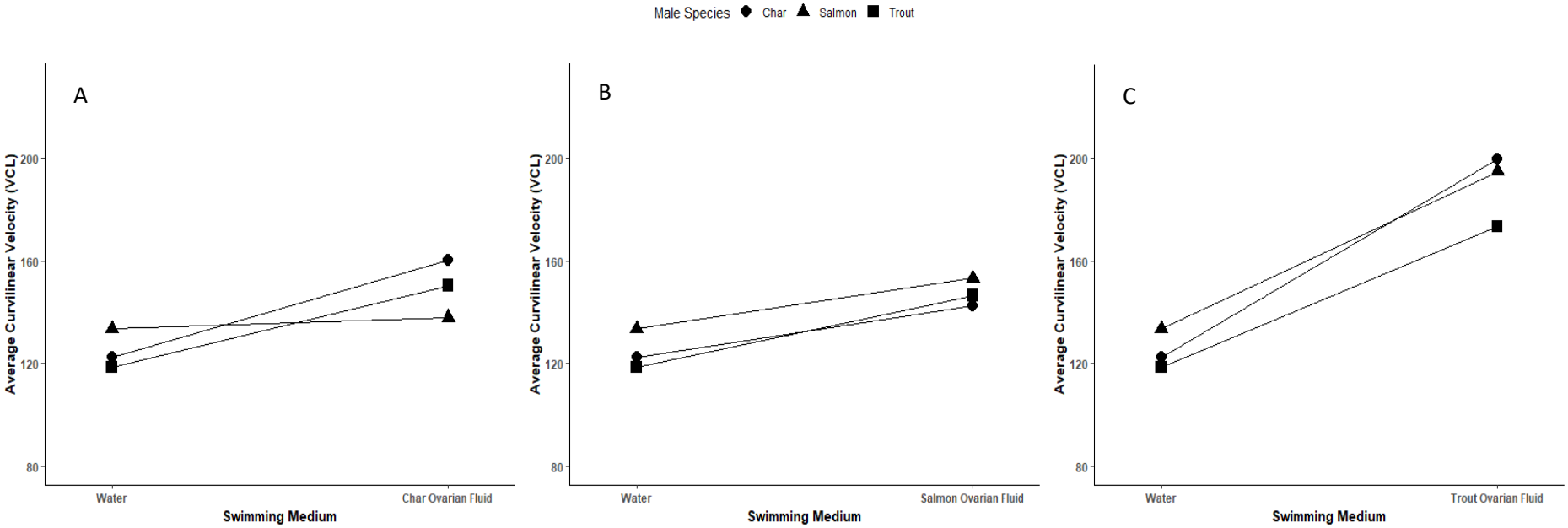
Appendix Figure 2-A7: Average VCL of sperm from replicate 7 males in water compared to ovarian fluid from the replicate 7 char (A), salmon (B), and trout (C) female. Circles represent char sperm, triangles represent salmon sperm, and squares represent trout sperm.



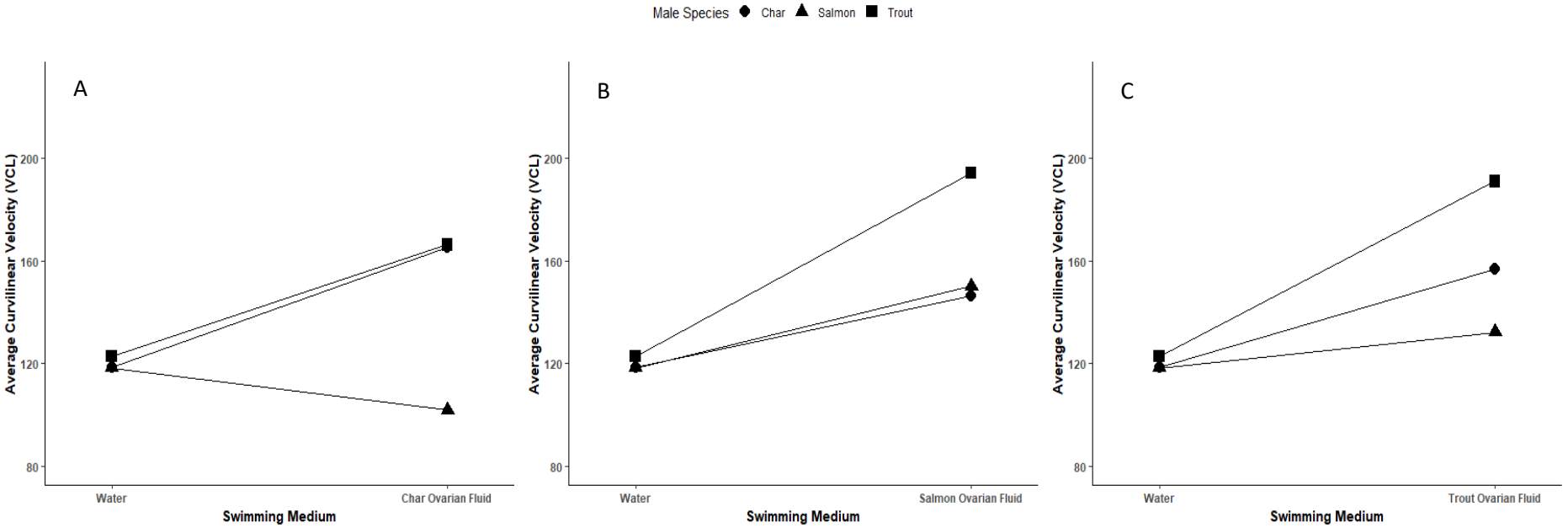
Appendix Figure 2-A8: Average VCL of sperm from replicate 8 males in water compared to ovarian fluid from the replicate 8 char (A), salmon (B), and trout (C) female. Circles represent char sperm, triangles represent salmon sperm, and squares represent trout sperm.



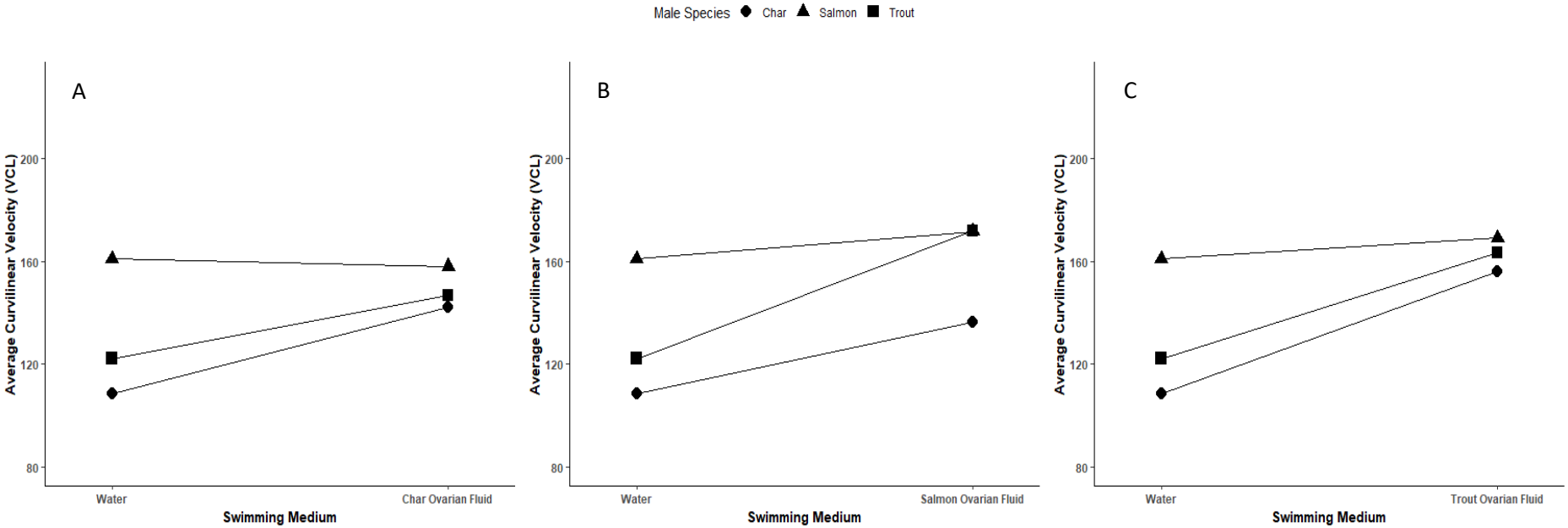
Appendix Figure 2-A9: Average VCL of sperm from replicate 9 males in water compared to ovarian fluid from the replicate 9 char (A), salmon (B), and trout (C) female. Circles represent char sperm, triangles represent salmon sperm, and squares represent trout sperm.



Appendix Figure 2-A10: Average VCL of sperm from replicate 10 males in water compared to ovarian fluid from the replicate 10 char (A), salmon (B), and trout (C) female. Circles represent char sperm, triangles represent salmon sperm, and squares represent trout sperm.

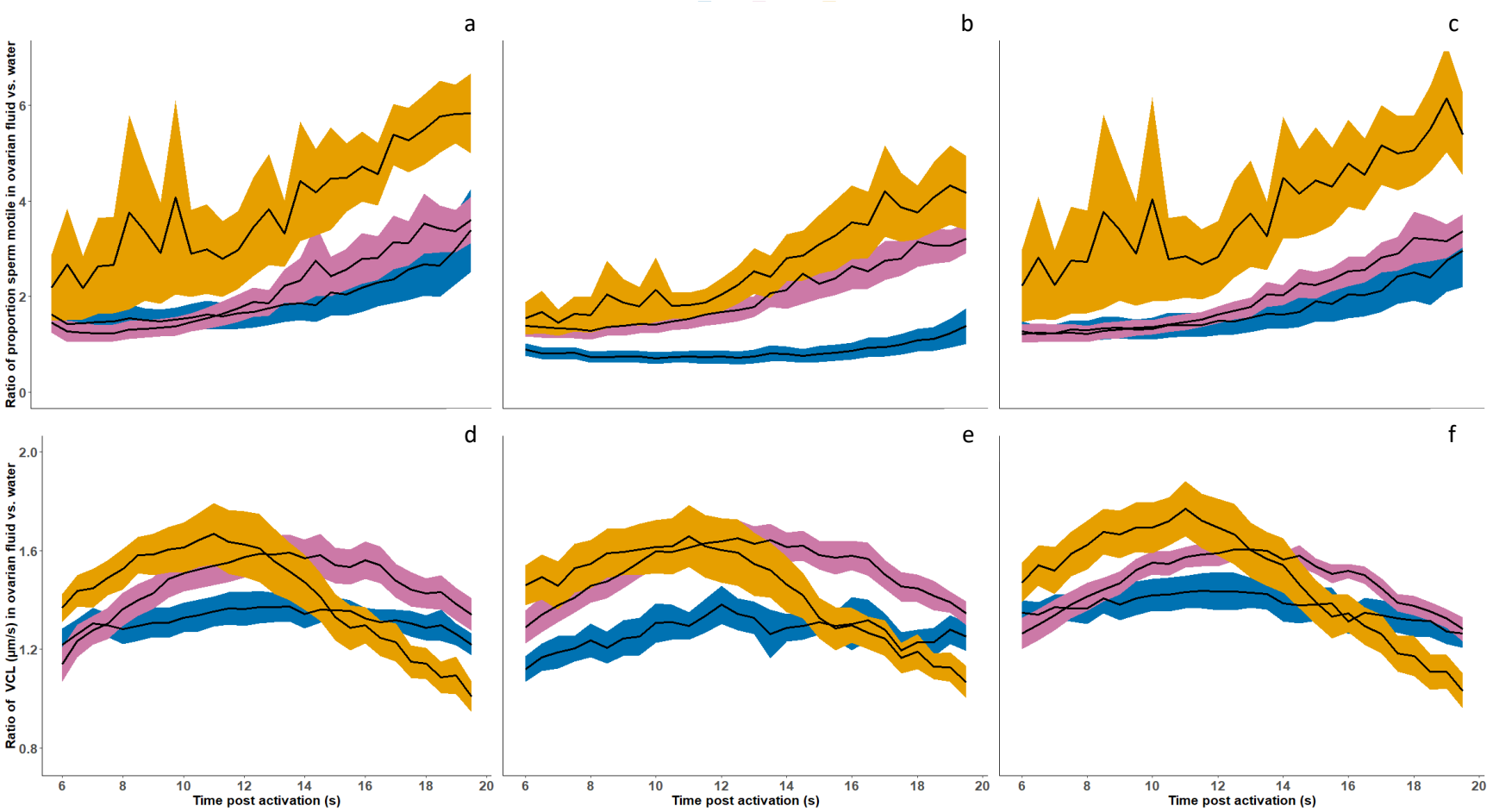


Appendix Figure 2-A11: Average VCL of sperm from replicate 11 males in water compared to ovarian fluid from the replicate 11 char (A), salmon (B), and trout (C) female. Circles represent char sperm, triangles represent salmon sperm, and squares represent trout sperm.



Appendix Figure 2-A12: Average VCL of sperm from replicate 12 males in water compared to ovarian fluid from the replicate 12 char (A), salmon (B), and trout (C) female. Circles represent char sperm, triangles represent salmon sperm, and squares represent trout sperm.

Appendix 2-B: Upregulation of motility and sperm swimming velocity from 6 to 30 seconds post activation



Appendix Figure 2B-1: Ratio of char (blue), salmon (pink), trout (orange) sperm motility and curvilinear velocity (VCL) in ovarian fluid compared to water. Each point shows sperm behaviour over 0.5 seconds. Black lines represent the average at each half second and colored bands represent the standard error for the 12 males within a species. Panels a b c show the ratio of sperm motility in water over char, salmon, and trout ovarian fluid, respectively. Panels d e f show the ratio of VCL in char, salmon, and trout, respectively.

Chapter 3

Cryptic female choice does not alter gamete-mediated paternal effects in hybridizing fish

Abstract

Post-mating sexual selection in the form of cryptic female choice provides opportunities for females to bias paternity to favor preferred males. However, little is known regarding how cryptic female choice might affect offspring outside of paternity through female mediated changes on sperm environmental experience. Gamete-mediated paternal effects are widespread, and female alteration of sperm experience may play an unrecognized role in shaping cryptic female choice. Using hybridizing fish from Newfoundland that have documented conspecific sperm preference mediated by differential upregulation of sperm swimming performance, we created artificial fertilizations under a randomized block, split-brood, and split-ejaculate design, to determine if sperm experience in different conditions influences offspring development. Prior to contact with eggs, sperm from each species experienced either short activation (immediate contact) or long activation (20s swimming delay) in either water, conspecific ovarian fluid, or heterospecific ovarian fluid. We predicted that effects from hybridization (sperm species) would be greater than those from sperm experience. We quantified hatch timing, hatchling size, and developmental stage several weeks after hatching and found that differential sperm experience created biologically irrelevant (average effect size of 1.4%) changes on offspring development, which were much smaller than the effects of hybridization itself (average effect size of 10.7%). Since ovarian fluid drastically changes sperm swimming behaviour when compared to water, we conclude that females in this population are able to modify paternity with cryptic female choice with no consequences to offspring development.

Introduction

Polygynandry, a mating system where females and males mate with several individuals in each reproductive episode, creates opportunities to expose eggs to multiple potential fathers of differential quality. This potentially allows females to be fertilized by higher quality fathers, which increases the chance of producing more fit offspring (Cothran, 2008; Garcia-Gonzalez & Simmons, 2005; Gowaty et al., 2010; Klemme et al., 2008). Polygynandry also opens avenues of post-mating sexual selection. In males, post-mating pre-zygotic sexual selection manifests as sperm competition where ejaculates from different individuals compete to fertilize the same egg (Birkhead & Pizzari, 2002; Parker & Pizzari, 2010). In addition to a rich array of pre-mating behaviors, females can bias the outcome of this competition after mating through alterations of sperm behavior and experience to favor certain males through cryptic female choice (Birkhead & Pizzari, 2002; Firman et al., 2017).

Cryptic female choice is taxonomically widespread, but mechanisms vary (Eberhard, 1996). For example, in internal fertilizers, females can mechanically reject ejaculates (birds, Pizzari & Birkhead, 2000; Wagner et al., 2004) or deposit preferred sperm in more favorable locations within the reproductive tract (*Drosophila*, Manier et al., 2013), while in external fertilizers chemical substances within fluids released with the eggs (referred to as egg water (Evans et al., 2012) and ovarian fluid (Zadmajid et al., 2019) in aquatic invertebrates and fishes respectively), change sperm swimming behaviour and influence sperm competition. Cryptic female choice is well known to strongly bias paternity through these changes in sperm swimming behaviour (Firman et al., 2017). While changing paternity drastically alters the

offspring's genotype, paternal effects create a change in gene expression. The examination of paternal effects is typically done through the lens of adult experience, e.g., diet (Evans et al., 2019; Purchase et al., 2021).

Additionally, there is increasing evidence that sperm environmental experience influences the development of offspring, which could also be considered paternal effects (Evans et al., 2019). Paternal effects occur when offspring are influenced by the father independent of a change in gene sequence (paternity) and can occur due to adult alterations to sperm quality or post-ejaculatory sperm experience. For example, in internal fertilizers such as mice (Bromfield et al., 2014) and pigs (Robertson, 2007), variations in the environment of the female reproductive tract and subsequent chemical interactions between ejaculates and the environment, have effects on offspring survival and phenotype. In species with sperm storage such as kittiwakes, older sperm (sperm from chronologically older matings) have negative effects on hatch success and offspring survival and condition (Wagner et al., 2004; White et al., 2008). For external fertilizers, factors such as the temperature and pH of the fertilization environment (Byrne & Przeslawski, 2013; Kekäläinen et al., 2018; Lymbery et al., 2020), and the time sperm swim before fertilization (Alavioon et al., 2017, 2019; Crean et al., 2012) influence offspring development. Since paternal effects are altered by environmental experience, and cryptic female choice drastically changes fertilization environment to change paternity, it follows those mechanisms of cryptic female choice should affect the individual sperm and lead to paternal effects. The question then is, does female modulated cryptic female choice impart paternal effects?

Given cryptic female choice changes paternity, which is generally compared among males of the same species, a conceptual default would be to test offspring development influences caused by cryptic female choice by examining female-male interactions within the same species as has recently been done in mussels (Lymbery et al., 2020). However, a more powerful approach is to examine hybridizing species because the strength of modifications to sperm performance should be stronger in the context of hybridization (Yeates et al., 2013). We did this with two hybridizing vertebrates, the first examination of female mediated paternal effects of this nature. Since polygynandrous matings sometimes include males from different species (Holman & Kokko, 2013), this is a valuable approach to take when examining the effects of cryptic female choice. This allows females to bias paternity to favor males of their own species through a process known as conspecific sperm preference (Howard, 1999). If cryptic female choice alters sperm in a way that influences offspring phenotype, we expect to see relatively large effect sizes in hybrid fertilizations because sperm are exposed to cues from a different species.

Conspecific sperm preference is seen in a wide range of taxa, including internal fertilizing terrestrial insects (Manier et al., 2013; Tyler et al., 2013) and birds (Cramer et al., 2016). For external fertilizers, females have less control over which males release sperm alongside her chosen mate but can regain control of paternity by altering sperm performance using chemicals that are released with eggs (Elofsson et al., 2006; Klibansky & McCartney, 2014). Unlike internal fertilization, it is comparatively easy for the researcher to manipulate the fertilization micro-environment using external fertilizers, as maternal effects can be easily controlled. This has been done and shown to affect offspring in mussels (Lymbery et al., 2020)

and fishes (Alavioon et al., 2017; Immler et al., 2014). We thus used two sister species of external fertilizing fish that readily hybridize to test the hypothesis that cryptic female choice alters the development of offspring outside of the effects of paternity.

Study system

Hybridization in salmonid fishes is common in the wild (Buss & Wright, 1958; Chevassus, 1979; Taylor, 2004). This is thought to be driven by high rates of polygynandry (Weir et al., 2010) and alternative reproductive tactics in the form of sneaker males circumventing pre-mating female choice and male competition (Garcia-Vazquez et al., 2002; McGowan & Davidson, 1992; Weir et al., 2016). Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) are closely related (Crête-Lafrenière et al., 2012), and hybrid matings and offspring are common in the wild (Álvarez & Garcia-Vazquez, 2011). Hybrids can also be easily produced with artificial fertilizations, thus making them an ideal study species to examine the effects of conspecific sperm preference on offspring.

In the genus *Salmo*, courtship behaviour helps females choose a preferred mate (Auld et al., 2019), but often multiple less preferred males release sperm into the nest as she spawns (Esteve, 2005). In some of these situations, the less preferred males are of the heterospecific sister species, but ovarian fluid released with eggs has been reported to upregulate swimming performance of conspecific sperm more than heterospecific sperm, leading to conspecific sperm preference and most eggs being fertilized by males of her own species (Yeates et al., 2013). This represents a strong female modification of the sperm experience and a potential source of a sperm phenotype alteration that might influence the development of offspring

(irrespective of paternity). Given the simple act of sperm swimming in water for 0s vs 20s prior to fertilization has been reported to change the development of Atlantic salmon embryos (Immler et al., 2014), we predicted this mechanism of cryptic female choice (the species of ovarian fluid that sperm swim in) alters offspring development via altering sperm experience.

Objectives and hypotheses

Our objective was to determine what role, if any, conspecific sperm preference in the face of hybridization plays in the expression of paternal effects. Since conspecific sperm preference and cryptic female choice changes the environment of fertilization as well as sperm experience, we postulate that these processes would then impart paternal effects. Specifically, we hypothesize that (1) effects of sperm swimming environment on offspring differs depending on whether sperm swim in conspecific or heterospecific ovarian fluid, that (2) sperm swimming environment will affect sperm from different species and their offspring in the same way, that (3) effects of sperm swimming environment on offspring differs depending on whether sperm swim in water or congeneric ovarian fluid, that (4) eggs fertilized by sperm that swim prior to fertilization hatch and develop faster than eggs fertilized by semen immediately after activation, and (5) effects resulting from hybridization (sperm species) and subsequently paternity should have a larger effect on the offspring than the paternal effects linked to differences in sperm swimming experience.

Methods

Experimental design

The experiment tested the influences of ovarian fluid on offspring development from individual males. Any response was thus based on intra-ejaculate changes (a split-ejaculate design, Purchase & Rooke, 2020). Critically, to reduce the influence of individual variation (Smith et al., 2019), female contributions were made from pools of fish from each species. We used a randomized block design whereby each block (n=6) of ovarian fluid came from a pool of equal proportions of three to four salmon and a separate pool of equal proportions from three to four trout and sperm (kept in isolation from one another) from one salmon and one trout. To trace offspring development, each block used a pool of equal proportions of eggs from three to four salmon, which were exposed to the sperm from the different treatments. Each traced group of sperm treated embryos were thus half-siblings (same father, one of three to four salmon mothers). The overall design thus combined split-ejaculates to control treatment, split-broods from pooled eggs to control individual variation in female quality, and a randomized block to allow for the collection of gametes and performing of artificial fertilizations on different days.

There were eight treatments within each block (Table 3-1). Sperm exposed to short activation in water (SAT-W, immediately added to eggs) and long activation in water (LAT-W, sperm swam 20 s before being added to eggs) prior to fertilization were used as an exact match to Immler et al. (2014), including the naming convention (Hypothesis 4). The other treatments (Table 3-1) were an elaboration on this design. These allowed us to determine if the medium in which sperm swim influences subsequent offspring development. Treatments LAT-SOF and LAT-TOF (Hypothesis 1) compared paternal effects on offspring from sperm that were exposed to salmon ovarian fluid (SOF, which was conspecific for salmon sperm (CSOF) and heterospecific

(HSOF) for trout sperm) and trout ovarian fluid (TOF, heterospecific for salmon sperm and conspecific for trout sperm). For Hypothesis 3, we combined data on SOF and TOF to compare LAT-W to LAT-OF in general.

Fish collection

Wild Atlantic salmon eggs were manually stripped from the Exploits River population in central Newfoundland, Canada (48.93 N, 55.67 W). Spawning adults were trapped in a fishway on Grand Falls on September 7 2018, and transferred to tanks indoors (198 cm diameter, 91 cm depth) on September 30, where they were maintained under ambient conditions, similar to Rooke et al. (2020). Prior to stripping, all fish were measured for standard length and tagged with individually coded plastic tags to ensure no fish was sampled twice, two weeks before gamete collection. Gametes were collected over a period of 14 days (but all gametes were collected on the same day for a given block) beginning in early November. For gamete stripping, salmon were anesthetized, paper toweled dry, and stripped for semen into plastic bags and eggs into glass jars. Care was taken to prevent urine and feces contamination. Semen and eggs were stored on ice and were immediately transported to laboratory facilities at Memorial University for experimentation which took place about 12 hours later.

Through coordinated efforts, for a given block, gametes were stripped from wild brown trout at the same time as those from salmon, using similar methods. Spawning trout were captured by dipnet in tributaries of Windsor Lake near St. John's Newfoundland, Canada (47.60 N, 52.78 W). These fish were introduced from Scotland in the 1880s (Westley & Fleming, 2011). Fish were measured for length and marked with a caudal fin clip to avoid repeat sampling of

individuals because collections of gametes occurred over multiple days. Although different anesthetics were used for both species, pre-gamete collection exposure to clove oil or MS-222 does not significantly affect sperm and egg function (Holcumb et al., 2004).

Fertilization

Samples were kept cool through all procedures, using a refrigerator and ice bath. Pooled salmon and trout eggs were strained with an aquarium net to remove ovarian fluid, which was mixed with 5°C tap water dechlorinated via air stone to create the desired treatments (Table 3-1). Trout eggs were discarded after ovarian fluid solutions were created. Next, salmon eggs were rinsed with nine ppt salt water to remove lingering ovarian fluid (Beirão et al., 2018). 150 microliters of semen were used in the short activation treatments (0s, SAT). Based on previous experiences by our lab, the sperm to egg ratio was low enough to avoid a ceiling effect (Beirão et al., 2018) but high enough to achieve a fertilization success that would produce adequate numbers of embryos. Following previous work (Alavioon et al., 2017; Immler et al., 2014), the amount of semen used in long activation treatments (20s, LAT) was doubled. After 20s, given ~50% of the sperm die naturally, doubling the amount of semen provided a similar sperm to egg ratio at fertilization for the SAT and LAT treatments, which may be important if there is egg selection (see Immler et al., 2014).

The fertilization procedure followed Immler et al. (2014). During short activation treatments, semen was put into the corner of a 50ml beaker, the 5°C water added, and the suspended, activated sperm were immediately poured onto 60 ml of eggs in a 250 ml beaker and mixed. In long activation treatments, the activating medium was poured onto the semen,

which was then left to sit for 20s before being poured over the eggs. Eggs and semen solution were gently mixed and left to rest for three minutes. Semen solution was then rinsed from the eggs using an aquarium net as a sieve. Eggs were then covered in fresh water and kept in a 5°C incubator in static beakers and left overnight to water harden.

Eggs that turned white during water hardening were deemed unviable and discarded the following morning. For each treatment (Table 3-1), 350 viable eggs were disinfected in a 1% ovadine solution, then separated equally into 7 PVC pipe incubation tubes (5.8 cm height x 5.8 cm diameter) with a screen on the bottom. Incubation tubes were transferred to one of three Marisource 4-tray vertical incubators. Each experimental block (n=6) was spread (Table 3-2) over 56 incubation tubes (8 treatments * 7 tubes * 50 embryos per tube) that were put into two incubation trays (each tray could hold 28 tubes). Salmon sperm and trout sperm tubes were split equally over both trays. In total there 2100 viable eggs incubated per treatment (eight) over all blocks (six).

Incubation

Incubators were placed in individual sump tanks containing chillers set to 5°C, inside a temperature-controlled room at 9°C. The two blocks in each incubator experienced slightly different water temperatures due to variation between the three incubators, but treatments within a block were always exposed to the same temperature. Water in the tanks was partially changed weekly before hatch and every two days thereafter to maintain water quality. Temperature measurements of the tanks and the room were taken hourly, and water quality measurements (pH, nitrate, nitrite, and ammonia) were taken weekly. Recirculating water was

passed through primary filtration, charcoal, and a UV sterilizer before entering the incubator at a flow rate of 16 L/min and gravity fed through the four incubation trays. Once the embryos reached approximately 20 accumulated temperature units (ATUs), any eggs/embryos that had turned white (Gaudemar and Beale, 1998) were removed weekly, except from 85-150 ATUs (~ two to four weeks after fertilization), to minimize disturbances to living embryos during this vulnerable life-history stage when gastrulation occurs (Battle, 1944; Tang et al., 1987).

To allow more time for paternal influence on offspring development (Eilertsen et al., 2009), a subset of eggs was checked at 240 ATUs (~ six weeks after fertilization) for the presence of embryos (signifying cell division). This indicated that fertilization success was low in some treatments (semen volume was purposefully restricted and was the likely cause). This was not done in a comprehensive way to report fertilization success to minimize the loss of critically important 800 ATU data. Hatch numbers are reported in Appendix 3-A. To compensate and prioritize standard developmental data collection across treatments before fish started to hatch at ~ 350 ATUs (~ ten weeks after fertilization), eggs were transferred from tube four and split equally into tubes one and seven (Table 3-2), to allow quantification of hatch times of embryos and an empty tube to store hatched individuals from other tubes. When embryos started to hatch within each treatment, individuals from tubes five and six from each treatment were counted daily and preserved in Stockard's solution (Murray & Beacham, 1986). Embryos that hatched from tubes two and three were transferred into the empty tube four daily to track individual hatch dates and increase the number of individuals available for analysis at 800 ATUs. Data were not collected from hatchlings in tubes one and seven until they reached 800 ATUs. If hatch success was lower than 25% for a treatment within a block, then all embryos within that

treatment were preserved at hatch. For the trout sperm LAT-W treatment, all embryos were taken at hatch due to low hatch success.

At hatch, salmonids are poorly developed and remain in gravel nests until ready to feed exogenously and emerge from the substrate (Mason, 1976). Unlike hatching, the timing of emergence can be somewhat subjective but generally occurs around 900 ATUs in Atlantic salmon (Gorodilov, 1996) as the endogenous yolk sac is almost completely consumed. We quantified development at 800 ATUs from fertilization (a precise number known for each embryo) which was ~ nine to 10 weeks after first hatching and ~ 22 weeks after fertilization. To reduce potential confounding variables on development post-hatch, hatchling densities were equalized at a maximum of 25 individuals per tube at approximately 575 ATUs (Table 3-2), roughly when all individuals completed hatching. Individuals were killed with an overdose of MS 222 at 800 ATUs and preserved in Stockard's solution for later analysis. In total, the experiment ran for 186 days.

Calculations and statistical analyses

The experiment was designed to test the influence of environmental conditions of sperm experience created by cryptic female choice on the development of offspring. To assess development, we quantified eight metrics. The timing to 50 % hatch (standardized to ATUs, Appendix 3-B), standard length (Appendix 3-C), head length (Appendix 3-D), and caudal ray count (Appendix 3-E) of embryos at hatch and the standard length (Appendix 3-F), the proportion of alevin with separated adipose fins (Appendix 3-G), the number of parr marks (Appendix 3-H) and caudal ray count (Appendix 3-I) of embryos at 800 ATUs from fertilization.

Because results were similar for all our metrics, of these 8, we report 4 in this manuscript, timing to 50% hatch, standard length at hatch, standard length at 800 ATUs, and the number of parr marks. These hatch metrics were chosen to replicate a previous study (Immler et al., 2013), while standard length at 800 ATUs was chosen to test for paternal effects at a longer timeframe than the previously cited study. Parr mark count was chosen over the remaining 800 ATU metrics as it appeared to be the next most relevant metric.

To calculate time to 50% hatch, each egg was given a binary number (0 = not hatched, 1= hatched) on each day. Hatch numbers were then used to create a logistic regression model relating day to hatch. Data collection stopped 1 week after the last embryo within a block hatched, as based on previous experiments in our lab, it was assumed that if no offspring hatched over a week, then no more offspring were likely to hatch. Equation 1 was solved for y = 0.5 to determine the day value where 50% of the offspring had hatched and then converted to ATUs (to ensure data was standardized across blocks).

$$0.5 = \frac{1}{1+e^{(-coefficient)*days-(intercept))}}.$$

Equation 1: Logistic regression equation. This was run for each block of each sperm experience treatment.

Embryos preserved at hatch were photographed with a Leica DFC420 camera mounted on a Leica M80 dissection microscope. Standard length measurements of the embryos were taken using ImageJ (Schneider et al., 2012). Embryos preserved at 800 ATUs were measured for standard length with the same process. Parr mark counts were conducted using the same

images. Effect sizes between treatments were created using mean values and the equation (1-X_2)/X_1. Where X represents the means for the appropriate treatments being compared. Heads were taken from a subset of individuals from each block (max of 50) for a different study. In situations where there were not enough individuals to measure standard length on intact samples due to poor hatch success in certain treatments, regressions for salmon and salmon-trout hybrid treatments were used to back-calculate intact length using standardized measurements from the first dorsal insertion to the caudal peduncle.

Several hypotheses were tested with the same modelling framework (Equation 2) whereby sperm species (salmon or trout), the tested treatments (see below), and their interaction were fixed effects, while block was a random effect. Treatments varied depending on the model being tested, so different data was used for each iteration of the model equation. To test the primary hypothesis that cryptic female choice affects the development of offspring, we compared the development of siblings that were fathered by sperm exposed to conspecific ovarian fluid (SOF for salmon sperm, TOF for trout sperm) to those exposed to heterospecific ovarian fluid (TOF for salmon sperm, SOF for trout sperm). Ovarian fluids were represented by the treatment term in the model (Hypothesis 1). Since Atlantic salmon and brown trout are very closely related, we hypothesized that the response of sperm experience would be similar for offspring sired by either species' sperm (Hypothesis 2), which was evaluated using the interaction term in equation 2.

$$DV = \beta_{Treatment} + \beta_{SpermSpecies} + \beta_{Treatment*SpermSpecies} + \beta_{Block} + \varepsilon$$

Equation 2: Model Equation. Data tested for each hypothesis was different depending on which treatments were compared. Error structure is not indicated because it varied for each model (see text).

To test the hypothesis that sperm experience in ovarian fluid in general influences subsequent offspring development, we compared data from embryos from the LAT-W treatment to data from embryos from the LAT-SOF and LAT-TOF treatments combined (LAT-OF) (Hypothesis 3). To retest the Immler et al. (2014) hypothesis that the simple act of sperm swimming influences offspring development, we use the same model to compare the data from treatments LAT-W and SAT-W (Hypothesis 4). Finally, we tested whether hybridization itself influenced development using the sperm species from equation 2 (Hypothesis 5). The final model did not include treatment as a term because we were testing all possible individuals. This also provided the ability to scale effect sizes from sperm experience treatments to that of the father species, i.e., hybridization.

We used a generalized linear mixed effects modelling approach to test for the effect of our treatments on offspring development. Models were tested using the 'lmertest package' (Kuznetsova et al., 2017) in R. Standard length at hatch and at 800 ATUs models were assumed to have a normal error structure and were tested using Type 3 ANOVAs. Time to hatch and parr mark count at 800 ATUs had a binomial and Poisson distribution, respectively, and were tested using Wald Chi-Square tests. Analysis of assumptions of parametric statistics revealed that our models for standard length at hatch and at 800 ATUs did not have a normal error structure. After transformation and other error structures did not meet this assumption, p-values around

our $\alpha=0.05$ at a threshold of $p=0.025<x<0.075$ were randomized to generate assumption free p-values (Ludbrook, 1994). All interactions, unless otherwise noted, were not significant at a threshold of $\alpha=0.05$.

Results

Hypotheses 1 and 2

We expected to find that offspring development would be altered through sperm swimming in conspecific (LAT-SOF-SS, LAT-TOF-TS) vs. heterospecific ovarian fluid (LAT-SOF-TS, LAT-TOF-SS) and that this pattern would be the same for salmon and trout (Figure 3-1). As expected, this pattern and those that followed were the same for both salmon and trout sperm, but offspring development was only slightly altered by differential ovarian fluid experience. There was no effect of conspecific ovarian fluid vs. heterospecific ovarian fluid on time to hatch (Figure 3-1a, Table 3-3, Table 3-4). For hatch size (Figure 3-1b), the interaction was statistically significant (Table 3-4). However, when the model was broken by sperm species, there was no significant difference in offspring from trout sperm but a slight effect of 1.3% in offspring from salmon sperm (Table 3-3, Table 3-4). At 800 ATUs, there were no significant differences in parr mark count (Figure 3-1d, Table 3-3, Table 3-4). The interaction was significant for body size (Figure 3-1c, Figure 3-4), but there was no effect in offspring from salmon sperm but a small 3% difference in offspring from trout sperm (Table 3-3). Extra metrics in the appendices (3-D, 3-E, 3-G, 3-I) show similar patterns.

Hypothesis 3

If ovarian fluid type is not important, we hypothesized that ovarian fluid, in general, would create strong effects when compared to sperm swimming in only water (LAT-W/LAT-OF). This was not the case (Figure 3-1). There was no effect on time to hatch (Figure 3-1a, Table 3-3, Table 3-4). For hatch size, the interaction was significant (Figure 3-1b, Table 3-4). However, the effect was in opposite directions for salmon sperm treatments (0.2%) and trout sperm treatments (1.6%). Nevertheless, both were extremely small (Table 3-3, Table 3-4). At 800 ATUs, trout sperm comparisons could not be made because too few hatched, but offspring size from salmon sperm was significantly affected by treatment (Figure 3-1c, Table 3-3, Table 3-4), although again, very small in magnitude. It is important to note that this statistical comparison does not include all blocks (Figure 3-1c, Table 3-3, Table 3-4), due to low hatch success in the LAT-W treatment. Even though the statistical comparison is significant (Table 3-4), we infer from the small effect size (0.19%) that it is not biologically relevant (Table 3-3). Lastly, there was no effect on parr mark count at 800 ATU (Figure 3-1d, Table 3-3, Table 3-4).

Hypothesis 4

Following Immler et al. (2014), we expected that the act of sperm swimming prior to fertilization in of itself affects offspring development (comparing SAT-W to LAT-W), however, we were unable to replicate their results. For time to 50 percent hatch and size at hatch, there was no significant effect of sperm swimming prior to fertilization (Figure 3-1a and 3-1b, Table 3-3, Table 3-4). At 800 ATUs, trout sperm comparisons could not be made due to poor hatch success, but offspring size from salmon sperm was significantly different but not biologically relevant (0.4 %), and parr mark count was not different (Figure 3-1c and 3-1d, Table 3-3, Table 3-4).

Hypothesis 5

As expected, hybridization influenced the development of embryos derived from salmon eggs more than paternal effects did. In all cases, sperm species was significant (Table 3-4) with greater effect size than any of the sperm swimming treatments (Table 3-3). Split-brood salmon eggs hatched sooner (Figure 3-1a) and at larger sizes (Figure 3-1b) and continued to show faster development at 800 ATUs (Figure 3-1c and Figure 3-1d) when hybridized by brown trout sperm.

Discussion

We sought to determine if cryptic female choice influences offspring development independent of paternity by evaluating the effects of chemically mediated conspecific sperm preference in the context of hybridizing fish. This is the first time parental effects have been analyzed in the context of hybridization and the first time gamete-mediated paternal effects have been examined in vertebrates in the context of cryptic female choice. We found that sperm experience in conspecific ovarian fluid vs. heterospecific ovarian fluid did not strongly alter embryo development. More surprisingly, neither did sperm experience in any ovarian fluid vs. water treatments, nor whether sperm swam prior to contact with eggs. In the previous chapter, we found that sperm experience was heavily modified by exposure to ovarian fluid vs. water, but in this chapter we found no significant effect in terms of non-genetic alterations to offspring. This implies that cryptic female choice is free to modify paternity without consequences to embryo development caused by gamete-mediated paternal effects.

As expected, hybridization did strongly influence development, as salmon eggs fathered by trout sperm hatched faster and larger than pure-species siblings. This pronounced difference

continued at 800 ATU, where hybrids were more developed at this stage. Since a change in paternity is a genetic change that affects both offspring genotype and phenotype; it stands to reason that a change in the father's species would produce such drastic differences. The effects of hybridization were much larger than those of environmentally mediated paternal effects through experience in ovarian fluid or the time sperm swam before fertilization.

What remains puzzling is why we were unable to replicate the findings in the Immler et al. (2014) study despite employing similar experimental procedures. Immler et al. (2014) found that longer sperm swimming times prior to contact with eggs was linked to shorter hatch times of offspring and larger sizes of offspring at hatch. Similarly, the same research group found that the overall fitness and survival of zebrafish offspring was increased by longer swimming sperm time (Alavioon et al., 2017, 2019). In the original salmon study, Immler et al. (2014) measured timing of hatch, embryo size at hatch, and size at three weeks after hatch. While we used similar parameters at hatch in our study, our fish were reared longer (800 ATUs, ~ nine to 10 weeks after first hatch). While Immler et al. (2014) concluded sperm swimming for 20s prior to fertilization (LAT) causes embryos to hatch faster, we found no functional effect, and non-significant trends moving in the opposite direction for the two species. The effect size of the 20s delay on the ejaculate may not be the same between the studies because our study used a temperature of 5°C vs 6.7°C. However, this should not significantly affect the robustness of our study as Immler et al. (2014) reported the most significant changes to offspring at 20s. This could also be because we used a different population of Atlantic salmon that had been isolated from the Immler et al. (2014) salmon for at least 600,000 years (Lehnert et al., 2020). For instance, these fish have differing numbers of chromosomes (Hartley, 1987) or perhaps

differing populations have differing sperm mortalities (e.g. Immler et al. 2014 reported 50% of sperm dead at 20s).

It is possible that the isolation between European and North American salmon may have something to do with our lesser than expected effect of cryptic female choice. Newfoundland Atlantic salmon have only reproductively interacted with introduced and invasive brown trout for ~ 140 years (Hustins, 2007; Westley & Fleming, 2011). Also, our sample populations have not had the chance to reproductively interact. As of this writing, brown trout have not invaded the Exploits River watershed where our salmon originated (Westley & Fleming, 2011). Given that these fish do naturally hybridize in Newfoundland where brown trout are present (McGowan & Davidson, 1992), we expected that these cryptic female choice mechanisms would be present as well. The fact that these fish are reproductively isolated may have something to do with the apparent weakness of these effects on sperm swimming (see Chapter 2) and suggest sympatric populations should be tested to determine if these paternal effects are still weak.

There are other mechanisms of cryptic female choice among internal fertilizers that we know have stronger effects on offspring development than those in external fertilizers. For example, in *Drosophila*, differing placement of sperm in the female's reproductive tract results in a paternal effect derived from how far the sperm must swim to fertilize the egg (Manier et al., 2013). In black-legged kittiwakes (*Rissa tridactylyus*), females eject older ejaculates in their reproductive tract in favor of newer ejaculates (Wagner et al., 2004), which is known to strongly affect offspring phenotype (White et al., 2008). In these strong examples, sperm experience is likely to drastically vary between males and within ejaculates, which could increase the strength

or prevalence of gamete mediated paternal effects. Due to the diversity of cryptic female choice mechanisms and hybridization across taxa, we recommend conducting other studies in other systems with robust cryptic female choice to fully understand the role females can play in influencing paternal effects.

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Literature cited

Alavioon, G., Garcia, A. C., LeChatelier, M., Maklakov, A. A., & Immler, S. (2019). Selection for longer lived sperm within ejaculate reduces reproductive ageing in offspring. *Evolution Letters*, *3*(2), 198–206. https://doi.org/10.1002/evl3.101

Alavioon, G., Hotzy, C., Nakhro, K., Rudolf, S., Scofield, D. G., Zajitschek, S., Maklakov, A. A., & Immler, S. (2017). Haploid selection within a single ejaculate increases offspring fitness. *Proceedings of the National Academy of Sciences*, *114*(30), 8053–8058. https://doi.org/10.1073/pnas.1705601114

Álvarez, D., & Garcia-Vazquez, E. (2011). Maintenance of asymmetric hybridization between Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) via postzygotic barriers and paternal effects. *Canadian Journal of Fisheries and Aquatic Sciences, 68*(4), 593-602. https://doi.org/10.1139/f2011-004

Auld, H. L., Noakes, D. L., & Banks, M. (2019). Advancing mate choice studies in salmonids. *Reviews in Fish Biology and Fisheries, 29*, 249-276. https://doi.org/10.1007/s11160-019-09551-5

Battle, H. I. (1944). The embryology of the Atlantic salmon (*Salmo salar*). *Canadian Journal of Research, 22d*(5), 105-125. https://doi.org/10.1139/cjr44d-008

Beirão, J., Litt, M. A., & Purchase, C. F. (2018). Chemically-dispersed crude oil and dispersant affects sperm fertilizing ability, but not sperm swimming behaviour in capelin (*Mallotus villosus*). *Environmental Pollution*, *241*, 521–528. https://doi.org/10.1016/j.envpol.2018.05.080

Birkhead, T. R., & Pizzari, T. (2002). Postcopulatory sexual selection. *Nature Reviews. Genetics*, *3*(4), 262–273. https://doi.org/10.1038/nrg774

Bromfield, J. J., Schjenken, J. E., Chin, P. Y., Care, A. S., Jasper, M. J., & Robertson, S. A. (2014). Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proceedings of the National Academy of Sciences*, *111*(6), 2200–2205. https://doi.org/10.1073/pnas.1305609111

Buss, K., & Wright, J. E. (1958). Appearance and fertility of trout hybrids. *Transactions of the American Fisheries Society*, *87*(1), 172–181. https://doi.org/10.1577/1548-8659(1957)87[172:AAFOTH]2.0.CO;2

Byrne, M., & Przeslawski, R. (2013). Multistressor impacts of warming and acidification of the ocean on marine invertebrates' life histories. *Integrative and Comparative Biology*, *53*(4), 582–596. https://doi.org/10.1093/icb/ict049

Chevassus, B. (1979). Hybridization in salmonids: Results and perspectives. *Aquaculture*, *17*(2), 113–128. https://doi.org/10.1016/0044-8486(79)90047-4

Cothran, R. D. (2008). Direct and indirect fitness consequences of female choice in a crustacean. *Evolution*, *62*(7), 1666–1675. https://doi.org/10.1111/j.1558-5646.2008.00403.x

Cramer, E. R. A., Ålund, M., McFarlane, S. E., Johnsen, A., & Qvarnström, A. (2016). Females discriminate against heterospecific sperm in a natural hybrid zone. *Evolution*, *70*(8), 1844–1855. https://doi.org/10.1111/evo.12986

Crean, A. J., Dwyer, J. M., & Marshall, D. J. (2012). Fertilization is not a new beginning: The relationship between sperm longevity and offspring performance. *PLoS ONE*, *7*(11), e49167. https://doi.org/10.1371/journal.pone.0049167

Crête-Lafrenière, A., Weir, L. K., & Bernatchez, L. (2012). Framing the Salmonidae family phylogenetic portrait: A more complete picture from increased taxon sampling. *PLoS ONE*, *7*(10). https://doi.org/10.1371/journal.pone.0046662

Eberhard, W. G. (1996). *Female Control: Sexual Selection by Cryptic Female Choice* (Vol. 69). Princeton University Press. https://doi.org/10.2307/j.ctvs32rx1

Elofsson, H., Look, K. J. W. V., Sundell, K., Sundh, H., & Borg, B. (2006). Stickleback sperm saved by salt in ovarian fluid. *Journal of Experimental Biology*, *209*(21), 4230–4237. https://doi.org/10.1242/jeb.02481

Esteve, M. (2005). Observations of spawning behaviour in Salmoninae: *Salmo, Oncorhynchus* and *Salvelinus*. *Reviews in Fish Biology and Fisheries*, *15*, 1–21. https://doi.org/10.1007/s11160-005-7434-7

Evans, J. P., Garcia-Gonzalez, F., Almbro, M., Robinson, O., & Fitzpatrick, J. L. (2012). Assessing the potential for egg chemoattractants to mediate sexual selection in a broadcast spawning marine invertebrate. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1739), 2855–2861. https://doi.org/10.1098/rspb.2012.0181

Evans, J. P., Wilson, A. J., Pilastro, A., & Garcia-Gonzalez, F. (2019). Ejaculate-mediated paternal effects: Evidence, mechanisms and evolutionary implications. *Reproduction*, *157*(4), R109–R126. https://doi.org/10.1530/REP-18-0524

Firman, R. C., Gasparini, C., Manier, M. K., & Pizzari, T. (2017). Postmating female control: 20 years of cryptic female choice. *Trends in Ecology & Evolution*, *32*(5), 368–382. https://doi.org/10.1016/j.tree.2017.02.010

Garcia-Gonzalez, F., & Simmons, L. (2005). The evolution of polyandry: Intrinsic sire effects contribute to embryo viability. *Journal of Evolutionary Biology*, *18*(4), 1097-1103 https://doi.org/10.1111/j.1420-9101.2005.00889.x

Garcia-Vazquez, E., Moran, P., Perez, J., Martinez, J. L., Izquierdo, J. I., de Gaudemar, B., & Beall, E. (2002). Interspecific barriers between salmonids when hybridisation is due to sneak mating. *Heredity*, *89*(4), 288–292. https://doi.org/10.1038/sj.hdy.6800134

Gaudemar, B. de, & Beall, E. (1998). Effects of overripening on spawning behaviour and reproductive success of Atlantic salmon females spawning in a controlled flow channel. *Journal of Fish Biology*, *53*(2), 434–446. https://doi.org/10.1111/j.1095-8649.1998.tb00991.x

Gorodilov, Y. N. (1996). Description of the early ontogeny of the Atlantic salmon, (*Salmo salar*), with a novel system of interval (state) identification. *Environmental Biology of Fishes*, *47*(2), 109–127. https://doi.org/10.1007/BF00005034

Gowaty, P. A., Kim, Y.-K., Rawlings, J., & Anderson, W. W. (2010). Polyandry increases offspring viability and mother productivity but does not decrease mother survival in *Drosophila pseudoobscura*. *Proceedings of the National Academy of Sciences*, *107*(31), 13771–13776. https://doi.org/10.1073/pnas.1006174107

Holcomb, M., Woolsey, J., Cloud, J. G., & Ingermann, R. L. (2004). Effects of Clove Oil, Tricaine, and CO2 on Gamete Quality in Steelhead and White Sturgeon. *North American Journal of Aquaculture*, *66*(3), 228–233. https://doi.org/10.1577/A03-027.1

Holman, L., & Kokko, H. (2013). The consequences of polyandry for population viability, extinction risk and conservation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1613), 20120053. https://doi.org/10.1098/rstb.2012.0053

Howard, D. J. (1999). Conspecific sperm and pollen precedence and speciation. *Annual Review of Ecology and Systematics*, *30*(1), 109–132. https://doi.org/10.1146/annurev.ecolsys.30.1.109

Hustins, D. (2007). Brown trout and rainbow trout: A journey Into Newfoundland waters. Tight Lines Publishers.

Immler, S., Hotzy, C., Alavioon, G., Petersson, E., & Arnqvist, G. (2014). Sperm variation within a single ejaculate affects offspring development in Atlantic salmon. *Biology Letters*, *10*(2), 20131040. https://doi.org/10.1098/rsbl.2013.1040

Kekäläinen, J., Oskoei, P., Janhunen, M., Koskinen, H., Kortet, R., & Huuskonen, H. (2018). Sperm pre-fertilization thermal environment shapes offspring phenotype and performance. *The Journal of Experimental Biology*, *221*(20). https://doi.org/10.1242/jeb.181412

Klemme, I., Ylönen, H., & Eccard, J. A. (2008). Long-term fitness benefits of polyandry in a small mammal, the bank vole *Clethrionomys glareolus*. *Proceedings of the Royal Society B: Biological Sciences*, *275*(1638), 1095–1100. https://doi.org/10.1098/rspb.2008.0038

Klibansky, L. K. J., & McCartney, M. A. (2014). Conspecific sperm precedence is a reproductive barrier between free-spawning marine mussels in the northwest Atlantic *Mytilus* hybrid zone. *PLoS ONE*, *9*(9), e108433. https://doi.org/10.1371/journal.pone.0108433

Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2017). lmerTest Package: Tests in linear mixed effects models. *Journal of Statistical Software*, *82*(1), 1–26. https://doi.org/10.18637/jss.v082.i13

Lehnert, S. J., Kess, T., Bentzen, P., Clément, M., & Bradbury, I. R. (2020). Divergent and linked selection shape patterns of genomic differentiation between European and North American Atlantic salmon (*Salmo salar*). *Molecular Ecology*, *29*(12), 2160–2175. https://doi.org/10.1111/mec.15480

Ludbrook, J. (1994). Advantages of permutation (randomization) tests in clinical and experimental pharmacology and physiology. *Clinical and Experimental Pharmacology & Physiology*, *21*(9), 673–686. https://doi.org/10.1111/j.1440-1681.1994.tb02570.x

Lymbery, R. A., Berson, J. D., & Evans, J. P. (2020). Indirect parental effects on offspring viability by egg-derived fluids in an external fertilizer. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1940), 20202538. https://doi.org/10.1098/rspb.2020.2538

Lymbery, R. A., Evans, J. P., & Kennington, W. J. (2020). Post-ejaculation thermal stress causes changes to the RNA profile of sperm in an external fertilizer. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1938), 20202147. https://doi.org/10.1098/rspb.2020.2147

Manier, M. K., Lüpold, S., Belote, J. M., Starmer, W. T., Berben, K. S., Ala-Honkola, O., Collins, W. F., & Pitnick, S. (2013). Postcopulatory sexual selection generates speciation phenotypes in *Drosophila*. *Current Biology: CB*, *23*(19), 1853–1862. https://doi.org/10.1016/j.cub.2013.07.086

Mason, J. C. (1976). Some features of coho salmon, (*Oncorhynchus kisutch*) fry emerging from simulated redds and concurrent changes in photobehaviour. *Fishery Bulletin*, *74*(1). https://spo.nmfs.noaa.gov/content/some-features-coho-salmon-oncorhynchus-kisutch-fry-emerging-simulated-redds-and-concurrent

McGowan, C., & Davidson, W. S. (1992). Unidirectional natural hybridization between brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*) in Newfoundland. *Canadian Journal of Fisheries and Aquatic Sciences*, *49*(9), 1953–1958. https://doi.org/10.1139/f92-216

Murray, C., & Beacham, T. (1986). Effect of incubation density and substrate on the development of chum salmon eggs and alevins. *The Progressive Fish-Culturist*, *48*, 242–249. https://doi.org/10.1577/1548-8640(1986)48<242:EOIDAS>2.0.CO;2

Parker, G. A., & Pizzari, T. (2010). Sperm competition and ejaculate economics. *Biological Reviews of the Cambridge Philosophical Society*, *85*(4), 897–934. https://doi.org/10.1111/j.1469-185X.2010.00140.x

Pizzari, T., & Birkhead, T. R. (2000). Female feral fowl eject sperm of subdominant males. *Nature*, *405*(6788), 787–789. https://doi.org/10.1038/35015558

Purchase, C., Evans, J., & Roncal, J. (2021). Integrating natural and sexual selection across the biphasic life cycle. https://doi.org/10.32942/osf.io/eu3am

Purchase, C. F., & Rooke, A. C. (2020). Freezing ovarian fluid does not alter how it affects fish sperm swimming performance: Creating a cryptic female choice 'spice rack' for use in split-ejaculate experimentation. *Journal of Fish Biology*, *96*(3), 693–699. https://doi.org/10.1111/jfb.14263

Puterka, G. J. (2017). More virulent offspring result from hybridization of invasive aphid species, *Diuraphis noxia* (Hemiptera: Aphididae), with *Diuraphis tritici* endemic to the United States. *Journal of Economic Entomology*, *110*(2), 731–738. https://doi.org/10.1093/jee/tow301

Robertson, S. A. (2007). Seminal fluid signaling in the female reproductive tract: Lessons from rodents and pigs. *Journal of Animal Science*, *85*(13 Suppl), E36-44. https://doi.org/10.2527/jas.2006-578

Rooke, A. C., Palm-Flawd, B., & Purchase, C. F. (2020). The impact of a changing winter climate on the hatch phenology of one of North America's largest Atlantic salmon populations. *Conservation Physiology, 7*(1). https://doi.org/10.1093/conphys/coz015

Rosenfield, J. A., & Kodric-Brown, A. (2003). Sexual selection promotes hybridization between Pecos pupfish, (*Cyprinodon pecosensis)* and sheepshead minnow, (*Cyprinodon variegatus*). *Journal of Evolutionary Biology*, *16*(4), 595–606. https://doi.org/10.1046/j.1420-9101.2003.00557.x

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), 671–675. https://doi.org/10.1038/nmeth.2089

Sherman, C. D. H., Wapstra, E., & Olsson, M. (2010). Sperm competition and offspring viability at hybridization in Australian tree frogs, Litoria peronii and L. tyleri. *Heredity*, *104*(2), 141–147. https://doi.org/10.1038/hdy.2009.118

Smith, K., Byrne, M., Deaker, D., Hird, C., Nielson, C., Wilson-McNeal, A., & Lewis, C. (2019). Sea urchin reproductive performance in a changing ocean: Poor males improve while good

males worsen in response to ocean acidification. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1907), 20190785. https://doi.org/10.1098/rspb.2019.0785

Tang, J., Bryant, M. D., & Brannon, E. L. (1987). Effect of temperature extremes on the mortality and development rates of coho salmon embryos and alevins. *The Progressive Fish-Culturist*, *49*(3), 167-174 https://www.tandfonline.com/doi/abs/10.1577/1548-8640%281987%2949%3C167%3AEOTEOT%3E2.0.CO%3B2

Taylor, E. B. 2004. Evolution in mixed company: evolutionary inferences from studies of natural hybridization in Salmonidae Pp. 232–263 in Hendry A. P., Stearns S. C., eds. Evolution illuminated: salmon and their relatives. Oxford University Press, Oxford. 510 p.

Tyler, F., Harrison, X. A., Bretman, A., Veen, T., Rodríguez-Muñoz, R., & Tregenza, T. (2013). Multiple post-mating barriers to hybridization in field crickets. *Molecular Ecology*, *22*(6), 1640–1649. https://doi.org/10.1111/mec.12187

Wagner, R. H., Helfenstein, F., & Danchin, E. (2004). Female choice of young sperm in a genetically monogamous bird. *Proceedings of the Royal Society B: Biological Sciences*, *271*(Suppl 4), S134–S137.

Weir, L. K., Breau, C., Hutchings, J. A., & Cunjak, R. A. (2010). Multiple paternity and variance in male fertilization success within Atlantic salmon (*Salmo salar*) redds in a naturally spawning population. *Journal of Fish Biology*, *77*(3), 479–493. https://doi.org/10.1111/j.1095-8649.2010.02690.x

Weir, L. K., Kindsvater, H. K., Young, K. A., & Reynolds, J. D. (2016). Sneaker males affect fighter male body size and sexual size dimorphism in salmon. *The American Naturalist*, *188*(2), 264–271. https://doi.org/10.1086/687253

Westley, P. A. H., & Fleming, I. A. (2011). Landscape factors that shape a slow and persistent aquatic invasion: Brown trout in Newfoundland 1883–2010. *Diversity and Distributions*, *17*(3), 566–579. https://doi.org/10.1111/j.1472-4642.2011.00751.x

White, J., Wagner, R., Helfenstein, F., Hatch, S., Mulard, H., Naves, L., & Danchin, E. (2008). Multiple deleterious effects of experimentally aged sperm in a monogamous bird. *Proceedings of the National Academy of Sciences*, *105*, 13947–13952. https://doi.org/10.1073/pnas.0803067105

Yeates, S. E., Diamond, S. E., Einum, S., Emerson, B. C., Holt, W. V., & Gage, M. J. G. (2013). Cryptic choice of conspecific sperm controlled by the impact of ovarian fluid on sperm swimming behavior. *Evolution*, *67*(12), 3523–3536. https://doi.org/10.1111/evo.12208

Zadmajid, V., Myers, J. N., Sørensen, S. R., & Ernest Butts, I. A. (2019). Ovarian fluid and its impacts on spermatozoa performance in fish: A review. *Theriogenology*, *132*, 144–152. https://doi.org/10.1016/j.theriogenology.2019.03.021

Chapter 3 tables

Table 3-1: Sperm experience treatments. Naming scheme followed Immler et al. (2014). Sperm from individual males were activated in 15 ml of swimming medium and then added to eggs either immediately (0s, short activation treatment) or after a 20s delay (long activation treatment). Ovarian fluid was pooled among three to four females in each block to create species level effects. Legend: SAT=Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF= Salmon ovarian fluid, TOF=Trout ovarian fluid, CSOF=Conspecific ovarian Fluid, HSOF=Heterospecific Ovarian Fluid SS=Salmon sperm, TS=Trout sperm.

Treatment	Time to Fertilization	Swimming Medium	Sperm Species
SAT-W-SS	0s	Water	Salmon
LAT-W-SS	20s	Water	Salmon
LAT-SOF-SS	20s	33% Salmon Ovarian Fluid (CSOF)	Salmon
LAT-TOF-SS	20s	33% Trout Ovarian Fluid (HSOF)	Salmon
SAT-W-TS	0s	Water	Trout
LAT-W-TS	20s	Water	Trout
LAT-SOF-TS	20s	33% Salmon Ovarian Fluid (HSOF)	Trout
LAT-TOF-TS	20s	33% Trout Ovarian Fluid (CSOF)	Trout

Table 3-2: Incubation tubes set up for each treatment in each block until 575 accumulated thermal units (ATUs). Embryos were equalized for density at a maximum of 25 individuals per tube at 575 ATUs. For treatments within blocks with poor hatch success, all embryos were preserved at hatch for timing and size measurements.

Tube Number	Procedure
1	Held hatchers until 575 ATUs, no data collected until 800 ATUs
2	Moved into empty tube 4 at hatch, data collected on hatch timing and size at 800 ATUs
3	Moved into empty tube 4 at hatch, data collected on hatch timing and size at 800 ATUs
4	Embryos were split into tubes 1 and 7 at 350 ATUs. Held hatchers from tubes 2 and 3 until 575 ATUs
5	Preserved at hatch for timing and size data.
6	Preserved at hatch for timing and size data.
7	Held hatchers until 575 ATUs, no data collected until 800 ATUs

Table 3-3: Effect sizes for sperm experience treatments and sperm species on the development of sibling embryos (Figure 3-1). * represents values significant at α=0.05 (Table 3-4). Effect sizes were calculated using the means of the treatments (1-Latter)/(Former) or the sperm species values (1-Trout/Salmon). Latter and former refer to order in the treatment column. Example for LAT-SOF/LAT-TOF effect size calculated as (1-LAT-TOF)/(LAT-SOF). Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Comparison	Dependent Variable	Effect Size Salmon Sperm	Effect Size Trout Sperm
LAT-SOF/LAT-TOF	Time H	0.01	-0.74
LAT-SOF/LAT-TOF	Stand H	-1.27*	0.91
LAT-SOF/LAT-TOF	Stand 800	0.08	-2.74*
LAT-SOF/LAT-TOF	Parr 800	3.27	-3.09
LAT-W/LAT-OF	Time H	-1.21	-0.82
LAT-W/LAT-OF	Stand H	-0.22*	1.62*
LAT-W/LAT-OF	Stand 800	0.19*	
LAT-W/LAT-OF	Parr 800	-2.98	
SAT-W/LAT-W	Time H	1.44	1.19
SAT-W/LAT-W	Stand H	1.03	-0.58
SAT-W/LAT-W	Stand 800	0.40*	
SAT-W/LAT-W	Parr 800	4.24	
Comparison	Dependent Variable	Effect Size	
Sperm species	Time H	-8.28*	
Sperm species	Stand H	6.28*	
Sperm species	Stand 800	5.83*	
Sperm species	Parr 800	22.30*	

Table 3-4: Results of statistical analysis for sperm experience treatments and sperm species for each variable. * represents values significant at α=0.05. Full model refers to models that included both salmon and trout sperm. If significant species interactions were found, the data were broken, and the model re-run for salmon sperm and trout sperm separately. Due to poor hatch success in some treatments full models were not possible for all combinations. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

			Full Model			Salmon Sperm Model			Trout Sperm Model		
Dependent Variable	**Comparison**	**Independent Variable**	F/χ^2	df	P	F/χ^2	df	P	F/χ^2	df	P
Time To Hatch	SAT-W/LAT-W	Sperm Species	1253	1	<0.001 *						
Time To Hatch	SAT-W/LAT-W	Swimming Medium	3.329	1	0.68						
Time To Hatch	SAT-W/LAT-W	Sperm Species*Swimming Medium	0.0763	1	0.782						
Time to Hatch	LAT-W/LAT-OF	Sperm Species	228.79	1	<0.001 *						
Time to Hatch	LAT-W/LAT-OF	Swimming Medium	2.72	1	0.099						
Time to Hatch	LAT-W/LAT-OF	Sperm Species*Swimming Medium	1.77	1	0.183						
Time to Hatch	LAT-SOF/LAT-TOF	Sperm Species	1026.709	1	<0.001 *						
Time to Hatch	LAT-SOF/LAT-TOF	Swimming Medium	1.109	1	0.2923						
Time to Hatch	LAT-SOF/LAT-TOF	Sperm Species*Swimming Medium	0.005	1	0.9437						
Standard Length at Hatch	SAT-W/LAT-W	Sperm Species	195.09	1	<0.001 *						
Standard Length at Hatch	SAT-W/LAT-W	Swimming Medium	0.76	1	0.206						
Standard Length at Hatch	SAT-W/LAT-W	Sperm Species*Swimming Medium	1.161	1	0.1181						
Standard Length at Hatch	LAT-W/LAT-OF	Sperm Species	247.516	1	<0.001 *						
Standard Length at Hatch	LAT-W/LAT-OF	Swimming Medium	0.675	1	0.411	4.33	1	0.035*	3.813	1	0.048*
Standard Length at Hatch	LAT-W/LAT-OF	Sperm Species*Swimming Medium	4.775	1	0.029						
Standard Length at Hatch	LAT-SOF/LAT-TOF	Sperm Species	642.222	1	<0.001 *						
Standard Length at Hatch	LAT-SOF/LAT-TOF	Swimming Medium	2.331	1	0.1271	18.323	1	<0.001 *	0.946	1	0.3314
Standard Length at Hatch	LAT-SOF/LAT-TOF	Sperm Species*Swimming Medium	17.122	1	<0.001 *						
Standard Length at 800 ATU	SAT-W/LAT-W	Sperm Species									
Standard Length at 800 ATU	SAT-W/LAT-W	Swimming Medium				9.4591	1	0.002 *			
Standard Length at 800 ATU	SAT-W/LAT-W	Sperm Species*Swimming Medium									
Standard Length at 800 ATU	LAT-W/LAT-OF	Sperm Species									
Standard Length at 800 ATU	LAT-W/LAT-OF	Swimming Medium				6.265	1	0.012 *			
Standard Length at 800 ATU	LAT-W/LAT-OF	Sperm Species*Swimming Medium									
Standard Length at 800 ATU	LAT-SOF/LAT-TOF	Sperm Species	483.313	1	<0.001 *						
Standard Length at 800 ATU	LAT-SOF/LAT-TOF	Swimming Medium	19.448	1	<0.001 *	1.205	1	0.246	25.763	1	<0.001 *
Standard Length at 800 ATU	LAT-SOF/LAT-TOF	Sperm Species*Swimming Medium	37.018	1	<0.001 *						
Parr Mark Count at 800 ATU	SAT-W/LAT-W	Sperm Species									
Parr Mark Count at 800 ATU	SAT-W/LAT-W	Swimming Medium				0.186	1	0.667			
Parr Mark Count at 800 ATU	SAT-W/LAT-W	Sperm Species*Swimming Medium									
Parr Mark Count at 800 ATU	LAT-W/LAT-OF	Sperm Species									
Parr Mark Count at 800 ATU	LAT-W/LAT-OF	Swimming Medium				0.1423	1	0.706			
Parr Mark Count at 800 ATU	LAT-W/LAT-OF	Sperm Species*Swimming Medium									
Parr Mark Count at 800 ATU	LAT-SOF/LAT-TOF	Sperm Species	66.52	1	<0.001 *						
Parr Mark Count at 800 ATU	LAT-SOF/LAT-TOF	Swimming Medium	1.433	1	0.23						
Parr Mark Count at 800 ATU	LAT-SOF/LAT-TOF	Sperm Species*Swimming Medium	1.097	1	0.295						

Chapter 3 figures

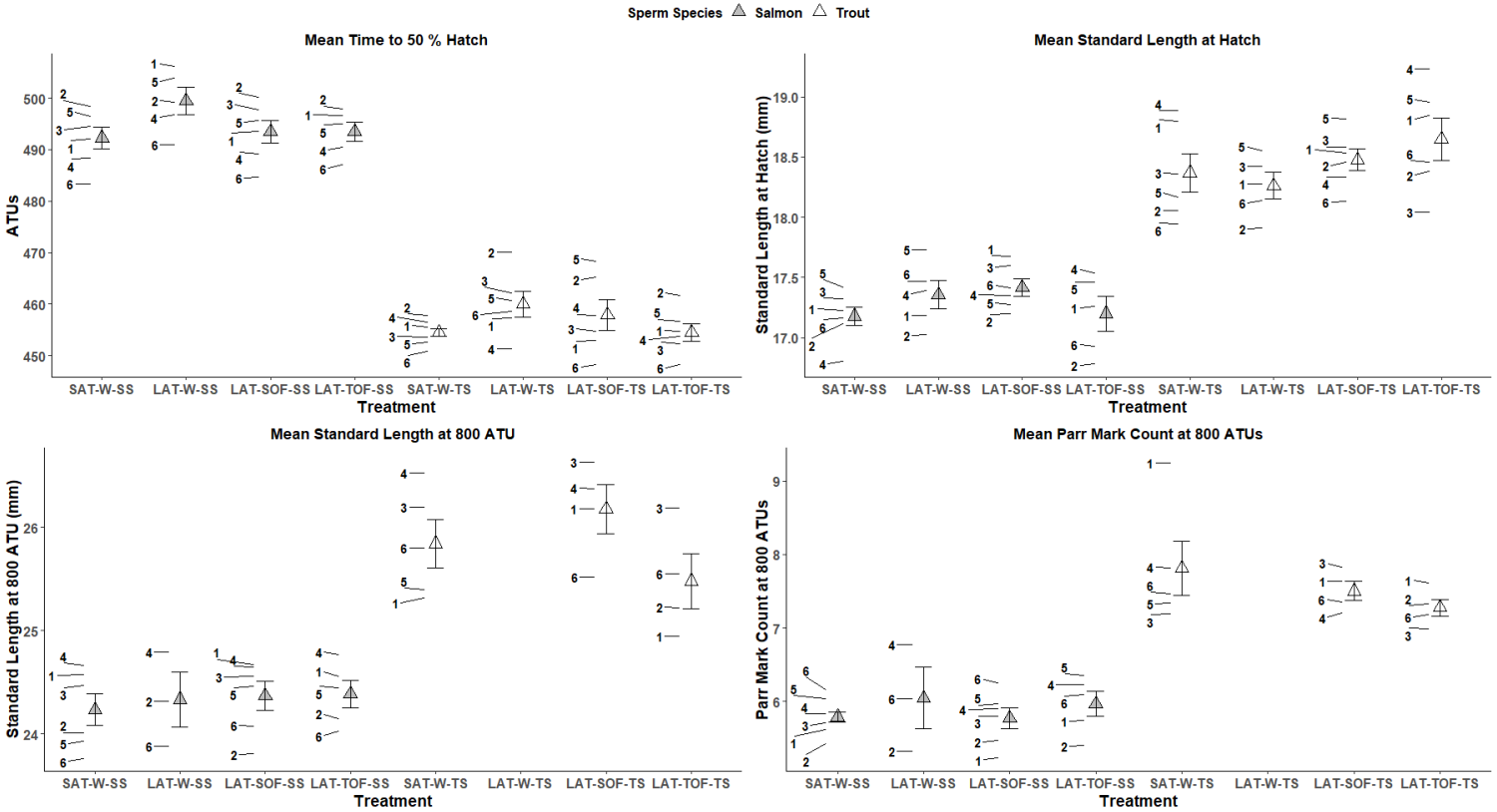


Figure 3-1: Developmental characteristics of Atlantic salmon (closed symbols) and hybrid salmon female (f) X brown trout male (m) embryos (open symbols) when sperm experienced different environmental treatments (Table 3-1) prior to contact with Atlantic salmon eggs. Each datum is the mean ± standard error among 6* blocks of parent fish. + represents our predicted value following the effect size derived from the Immler et al. (2014) study. Panels: (a) accumulated temperature units (ATUs) to 50% hatch, (b) standard length at hatch, (c) standard length at 800 ATUs, (d) parr mark count at 800 ATUs. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

*Poor hatch success in some treatments resulted in incomplete data at 800 ATUs (means for treatments LAT-W-SS and LAT-TOF-SS to be taken from five blocks, while no data were available for LAT-W-TS). Blocks are represented with numbers.

Chapter 3 appendices

Appendix 3-A: Hatch numbers by block

Appendix Table 3-A1: Block 1 alevin numbers. Percent hatch was taken from 350 eggs in all treatments except the LAT-SOF-TS treatment which was taken from 186 eggs. Incubation tube density was adjusted at approximately 567 ATU. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Treatment	Percent hatch	Number hatch	Number preserved at hatch	Dead alevin removed prior to density adjustment	Percent survival (hatch to density adjustment)	Number held to 800 ATU	Dead alevin post density adjustment	Percent survival after density adjustment	Total fish at 800 ATU
SAT-W-SS	71.71%	251	71	6	97.61%	174	18	88.46%	156
LAT-W-SS	1.14%	4	4	No data	No data	0	No data	No data	0
LAT-SOF-SS	88.86%	311	87	9	97.11%	215	33	81.87%	182
LAT-TOF-SS	73.14%	256	79	9	96.48%	168	30	78.26%	138
SAT-W-TS	17.14%	60	15	1	98.33%	44	0	100%	44
LAT-W-TS	2.29%	8	8	No data	No data	0	No Data	No data	0
LAT-SOF-TS	20.00%	70	30	2	97.14%	38	6	81.25%	32
LAT-TOF-TS	49.71%	174	52	0	100.00%	122	9	92.04%	113

Appendix Table 3-A2: Block 2 alevin numbers: Percent hatch was taken from 350 eggs. Incubation tube density was adjusted at approximately 567 ATU. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Treatment	Percent hatch success	Number hatch	Number preserved at hatch	Dead alevin removed pre density adjustment	Percent survival (hatch to density adjustment)	Number held to 800 ATU	Dead alevin post density adjustment	Percent survival after density adjustment	Total fish at 800 ATU
SAT-W-SS	81.14%	284	82	7	97.54%	195	7	96.28%	188
LAT-W-SS	64.00%	224	67	3	98.66%	154	6	95.95%	148
LAT-SOF-SS	57.71%	202	71	5	97.52%	126	14	87.5%	112
LAT-TOF-SS	80.86%	283	79	8	97.17%	196	16	91.11%	180
SAT-W-TS	10.29%	36	36	No Data	No Data	0	No Data	No data	0
LAT-W-TS	4.57%	16	16	No Data	No Data	0	No Data	No data	0
LAT-SOF-TS	3.71%	13	13	No Data	No Data	0	No Data	No data	0
LAT-TOF-TS	53.43%	187	60	3	98.4%	124	3	97.52%	121

Appendix Table 3-A3: Block 3 alevin numbers: Percent hatch was taken from 350 eggs. Incubation tube density was adjusted at approximately 567 ATU. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Treatment	Percent hatch success	Number hatch	Number preserved at hatch	Dead alevin removed pre density adjustment	Percent survival (hatch to density adjustment)	Number held to 800 ATU	Dead alevin post density adjustment	Percent survival after density adjustment	Total fish at 800 ATU
SAT-W-SS	48.86%	171	27	10	94.15%	134	18	84.48%	116
LAT-W-SS	0.00%	0	0	No data	No data	0	No data	No data	0
LAT-SOF-SS	68.00%	238	69	14	94.12%	155	14	90.07%	141
LAT-TOF-SS	0.00%	0	0	No data	No data	0	No data	No data	0
SAT-W-TS	57.14%	200	62	4	98.00%	134	3	97.71%	131
LAT-W-TS	3.43%	12	12	No data	No data	0	No data	No data	0
LAT-SOF-TS	35.71%	125	32	2	98.4%	91	4	95.40%	87
LAT-TOF-TS	63.14%	221	58	5	97.74%	158	1	99.36%	157

Appendix Table 3-A4: Block 4 alevin numbers: Percent hatch was taken from 350 eggs. Incubation tube density was adjusted at approximately 567 ATU. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Treatment	Percent hatch success	Number hatch	Number preserved at hatch	Dead alevin removed pre density adjustment	Percent survival (hatch to density adjustment)	Number held to 800 ATU	Dead alevin post density adjustment	Percent survival after density adjustment	Total fish at 800 ATU
SAT-W-SS	66.00%	231	64	12	94.81%	155	25	80.77%	130
LAT-W-SS	32.86%	115	38	6	94.78%	71	12	79.66%	59
LAT-SOF-SS	64.57%	226	53	10	95.58%	163	20	86.01%	143
LAT-TOF-SS	56.86%	199	53	11	94.47%	135	8	93.7%	127
SAT-W-TS	16.00%	56	17	2	96.43%	37	1	97.22%	36
LAT-W-TS	0.29%	1	1	No Data	No data	0	No data	No data	0
LAT-SOF-TS	48.57%	170	33	8	95.29%	129	4	96.8%	125
LAT-TOF-TS	3.71%	13	10	3	76.92%	0	No data	No data	0

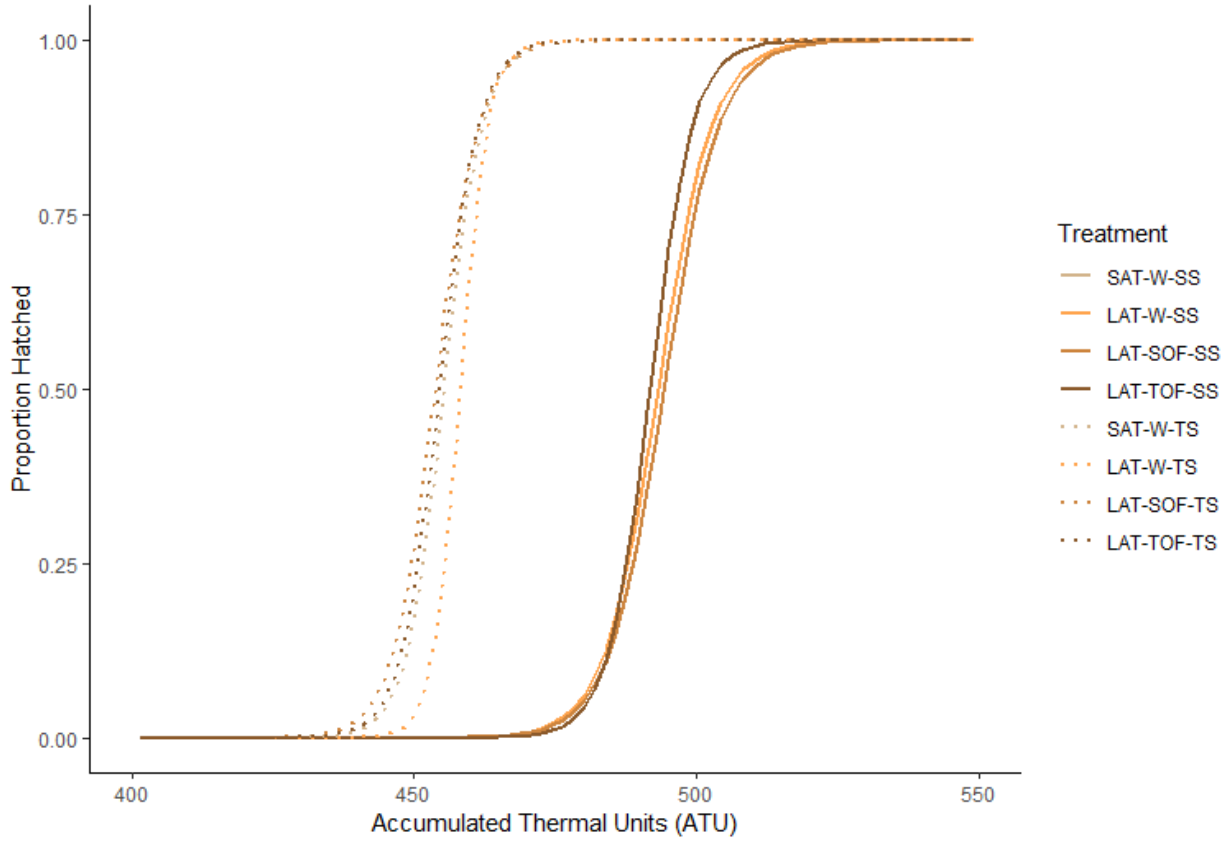
Appendix Table 3-A5: Block 5 alevin numbers: Percent hatch was taken from 350 eggs. Incubation tube density was adjusted at approximately 567 ATU. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Treatment	Percent hatch success	Number hatch	Number preserved at hatch	Dead alevin removed pre density adjustment	Percent survival (hatch to density adjustment)	Number held to 800 ATU	Dead alevin post density adjustment	Percent survival after density adjustment	Total fish at 800 ATU
SAT-W-SS	73.43%	257	65	18	93%	174	25	83.22%	149
LAT-W-SS	7.14%	25	22	3	88%	0	No data	No data	0
LAT-SOF-SS	75.14%	263	78	6	97.72%	179	21	86.71%	158
LAT-TOF-SS	80.86%	283	87	5	98.23%	191	20	88.3%	171
SAT-W-TS	52.57%	184	61	1	99.46%	122	3	2.52%	119
LAT-W-TS	1.71%	6	5	0	100%	1	0	97.48%	1
LAT-SOF-TS	0.29%	1	1	No data	No data	0	No data	No data	0
LAT-TOF-TS	4.00%	14	13	1	92.86%	0	No data	No data	0

Appendix Table 3-A6: Block 6 alevin numbers. Percent hatch was taken from 350 eggs. Incubation tube density was adjusted at approximately 567 ATU. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Treatment	Percent hatch success	Number hatch	Number preserved at hatch	Dead alevin removed	Percent survival (hatch to density adjustment)	Number held to 800 ATU	Dead alevin post density adjustment	Percent survival after density adjustment	Total fish at 800 ATU
SAT-W-SS	85.43%	299	85	21	92.98%	193	16	90.96%	177
LAT-W-SS	52.86%	185	45	8	95.68%	132	10	91.8%	122
LAT-SOF-SS	79.14%	277	83	18	93.5%	176	20	87.18%	156
LAT-TOF-SS	78.86%	276	81	10	96.38%	185	25	84.37%	160
SAT-W-TS	49.71%	174	46	8	95.4%	120	3	97.44%	117
LAT-W-TS	0.29%	1	1	0	0.00%	0	No data	No data	0
LAT-SOF-TS	68.29%	239	66	18	92.47%	155	7	95.27%	148
LAT-TOF-TS	55.43%	194	46	11	94.33%	137	6	95.42%	131

Appendix 3-B: Logistic regressions for time to 50 percent hatched by block



Appendix Figure 3-B1: Logistic regressions of hatch timing for block 1 (solid lines =Salmon x Salmon crosses, dashed =Salmon x Trout crosses, equation=$y = \frac{1}{1+e^{-coefficient)*ATU-(intercept)}}$). Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid. Sample sizes, intercepts, coefficients, and corresponding Pr(>|z|) values for each treatment were as follows.

SAT-W-SS n=142, -98.084 Pr(>|z|) =<0.001, 0.980 Pr(>|z|) =<0.001

LAT-W-SS n=4, -177.359 Pr(>|z|) =0.006, 1.726 Pr(>|z|) =0.006

LAT-SOF-SS n=173, -96.396 Pr(>|z|) =<0.001, 0.961 Pr(>|z|) =<0.001

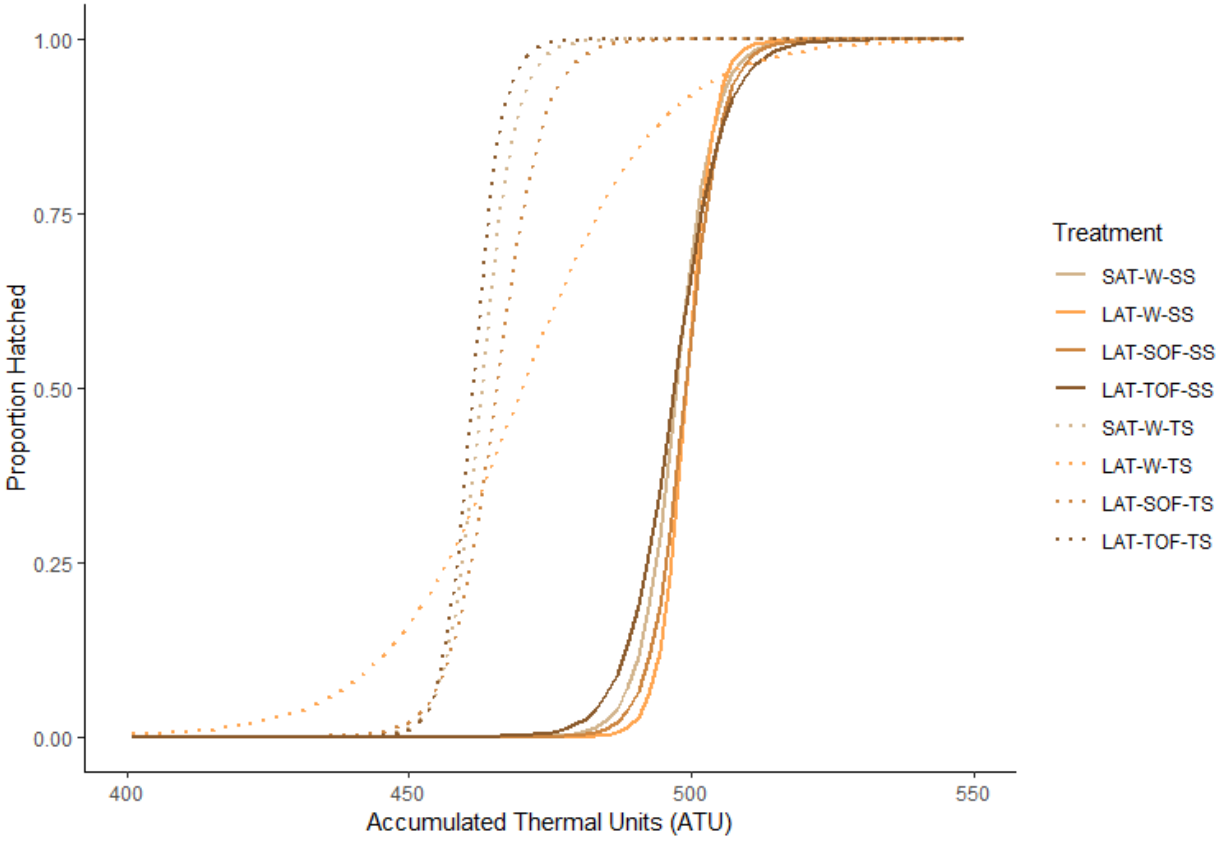
LAT-TOF-SS, n=153, -124.191 Pr(>|z|) =<0.001, 1.245 Pr(>|z|) =<0.001

SAT-W-TS n=52, -131.957 Pr(>|z|) =<0.001, 1.432 Pr(>|z|) =<0.001

LAT-W-TS n=8, -182.026 Pr(>|z|) =0.001, 1.963 Pr(>|z|) =0.001

LAT-SOF-TS n=67, -114.735 Pr(>|z|) =<0.001, 1.249 Pr(>|z|) =<0.001

LAT-TOF-TS n=97, -128.257 Pr(>|z|) =<0.001, 1.394 Pr(>|z|) =<0.001



Appendix Figure 3-B2: Logistic regressions of hatch timing for block 2 (solid lines =Salmon x Salmon crosses, dashed =Salmon x Trout crosses, equation=$y = \frac{1}{1+e^{-coefficient)*ATU-(intercept)}}$). Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid. Sample sizes, intercepts, coefficients, and corresponding Pr(>|z|) values for each treatment were as follows.

SAT-W-SS n=134, -144.055 Pr(>|z|) =<0.001, 1.424 Pr(>|z|) =<0.001

LAT-W-SS n=127, -200.134 Pr(>|z|) =<0.001, 1.972 Pr(>|z|) =<0.001

LAT-SOF-SS n=131, -152.024 Pr(>|z|) =<0.001, 1.498 Pr(>|z|) =<0.001

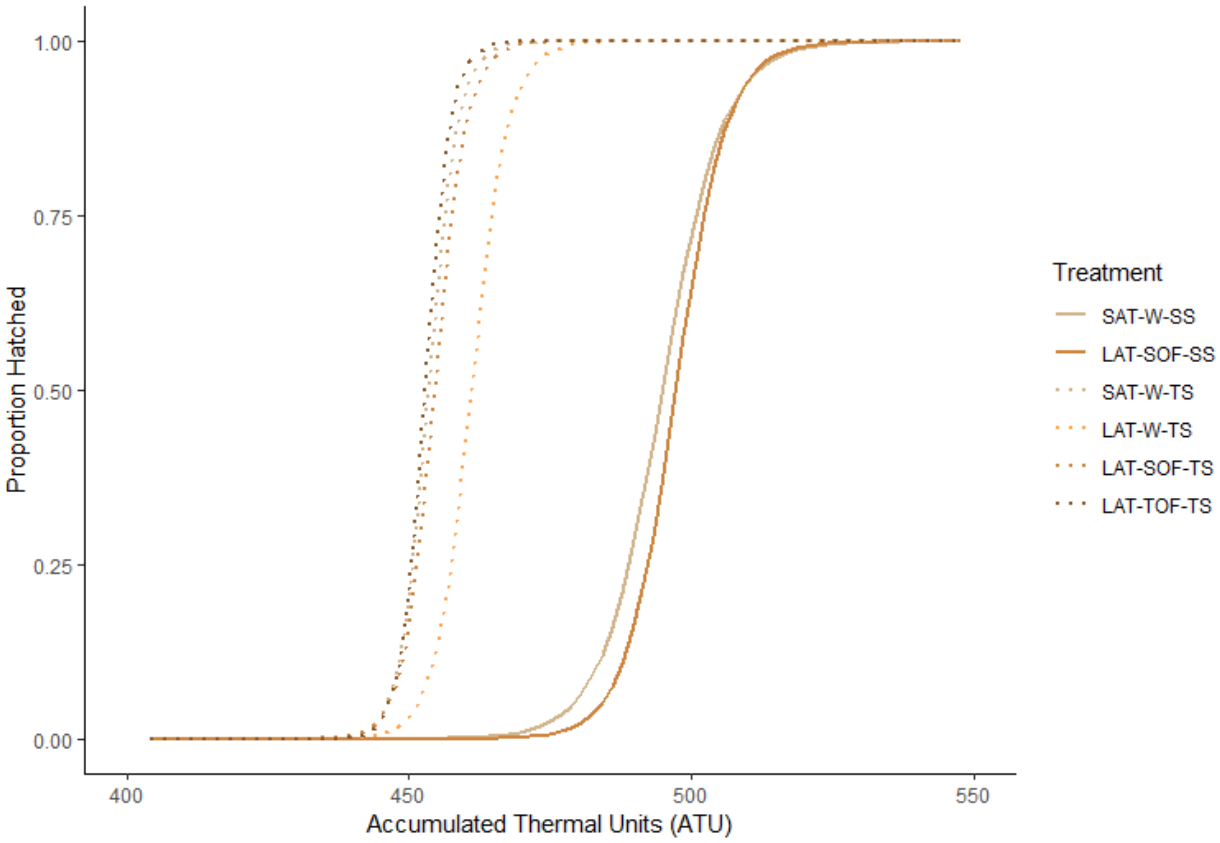
LAT-TOF-SS, n=163, -109.779 Pr(>|z|) =<0.001, 1.086 Pr(>|z|) =<0.001

SAT-W-TS n=36, -148.519 Pr(>|z|) =<0.001, 1.582 Pr(>|z|) =<0.001

LAT-W-TS n=16, -37.478 Pr(>|z|) =<0.001, 0.393 Pr(>|z|) =<0.001

LAT-SOF-TS n=13, =109.435 Pr(>|z|) =0.001, 1.159 Pr(>|z|) =0.001

LAT-TOF-TS n=107, -178.184 Pr(>|z|) =<0.001, 1.905 Pr(>|z|) =<0.001



Appendix Figure 3-B3: Logistic regressions of hatch timing for block 3 (solid lines =Salmon x Salmon crosses, dashed =Salmon x Trout crosses, equation=$y = \frac{1}{1+e^{-coefficient)*ATU-(intercept)}}$). Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid. Sample sizes, intercepts, coefficients, and corresponding Pr(>|z|) values for each treatment were as follows.

SAT-W-SS n=84, -90.930 Pr(>|z|) =<0.001, 0.833 Pr(>|z|) =<0.001

LAT-W-SS n=0

LAT-SOF-SS n=135, -103.213 Pr(>|z|) =<0.001, 0.940 Pr(>|z|) =<0.001

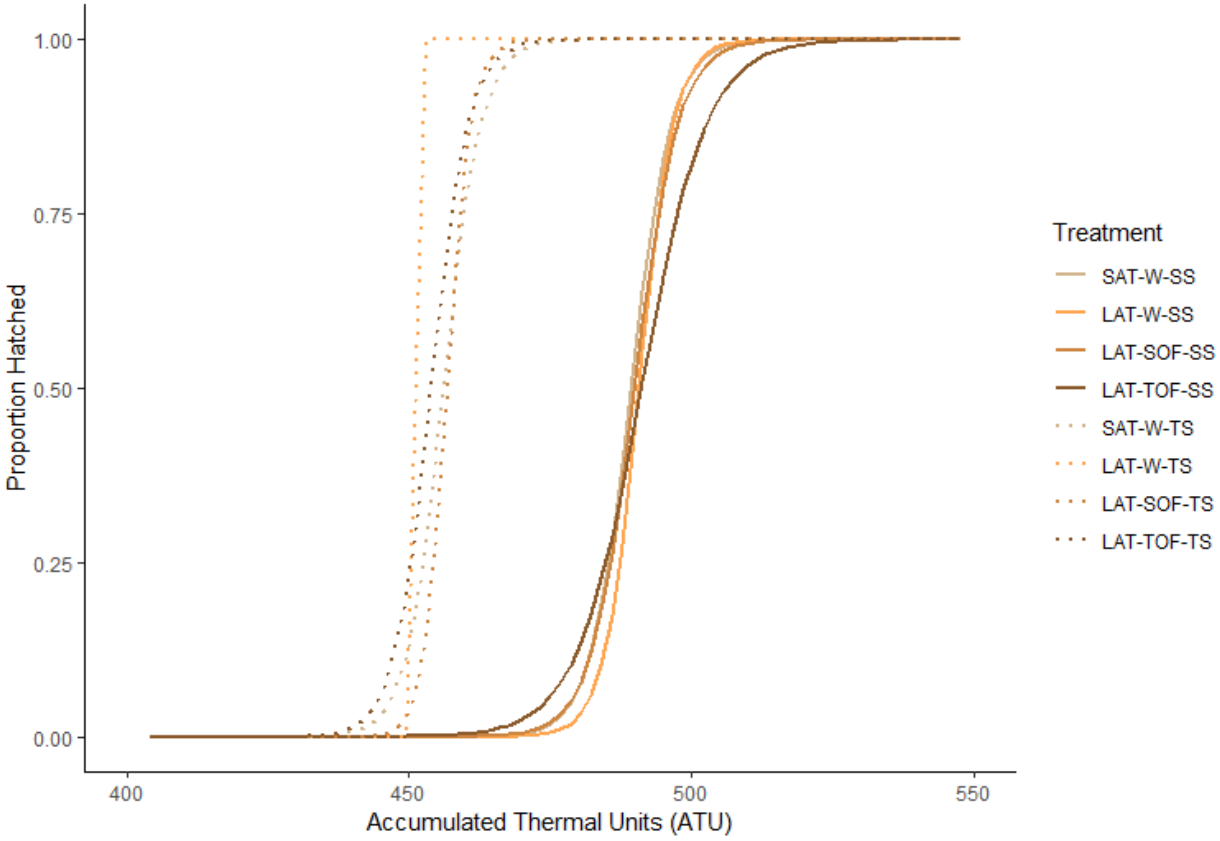
LAT-TOF-SS, n=0

SAT-W-TS n=117, -178.023 Pr(>|z|) =<0.001, 1.781 Pr(>|z|) =<0.001

LAT-W-TS n=12, -140.515 Pr(>|z|) =0.001, 1.382 Pr(>|z|) =0.001

LAT-SOF-TS n=64, -162.992 Pr(>|z|) =<0.001, 1.626 Pr(>|z|) =<0.001

LAT-TOF-TS n=112, -207.028 Pr(>|z|) =<0.001, 2.074 Pr(>|z|) =<0.001



Appendix Figure 3-B4: Logistic regressions of hatch timing for block 4 (solid lines =Salmon x Salmon crosses, dashed =Salmon x Trout crosses, equation=$y = \frac{1}{1+e^{-coefficient)*ATU-(intercept)}}$). Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid. Sample sizes, intercepts, coefficients, and corresponding Pr(>|z|) values for each treatment were as follows.

SAT-W-SS n=125, -134.571 Pr(>|z|) =<0.001, 1.246 Pr(>|z|) =<0.001

LAT-W-SS n=68, -158.789 Pr(>|z|) =<0.001, 1.467 Pr(>|z|) =<0.001

LAT-SOF-SS n=127, -125.559 Pr(>|z|) =<0.001, 1.161 Pr(>|z|) =<0.001

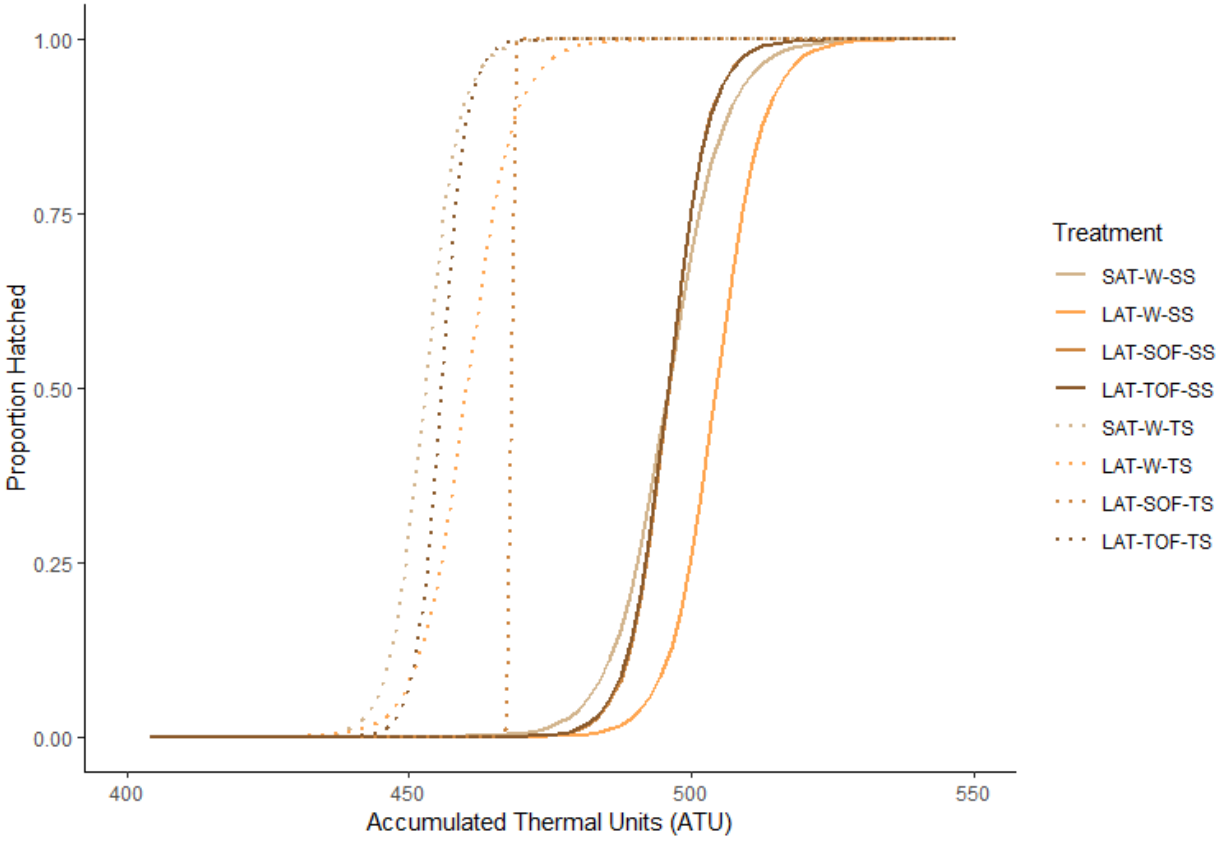
LAT-TOF-SS, n=112, -65.881 Pr(>|z|) =<0.001, 0.608 Pr(>|z|) =<0.001

SAT-W-TS n=32, -137.860 Pr(>|z|) =<0.001, 1.371 Pr(>|z|) =<0.001

LAT-W-TS n=1, -3105.240 Pr(>|z|) =0.988, 31.21 Pr(>|z|) =0.988

LAT-SOF-TS n=71, -216.635 Pr(>|z|) =<0.001, 2.151 Pr(>|z|) =<0.001

LAT-TOF-TS n=10, -136.988 Pr(>|z|) =0.001, 1.369 Pr(>|z|) =0.001



Appendix Figure 3-B5: Logistic regressions of hatch timing for block 5 (solid lines =Salmon x Salmon crosses, dashed =Salmon x Trout crosses, equation=$y = \frac{1}{1+e^{-coefficient)*ATU-(intercept)}}$). Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid. Sample sizes, intercepts, coefficients, and corresponding Pr(>|z|) values for each treatment were as follows.

SAT-W-SS n=133, -102.031 Pr(>|z|) =<0.001, 1.021 Pr(>|z|) =<0.001

LAT-W-SS n=22, -123.830 Pr(>|z|) =<0.001, 1.218 Pr(>|z|) =<0.001

LAT-SOF-SS n=154, -143.598 Pr(>|z|) =<0.001, 1.436 Pr(>|z|) =<0.001

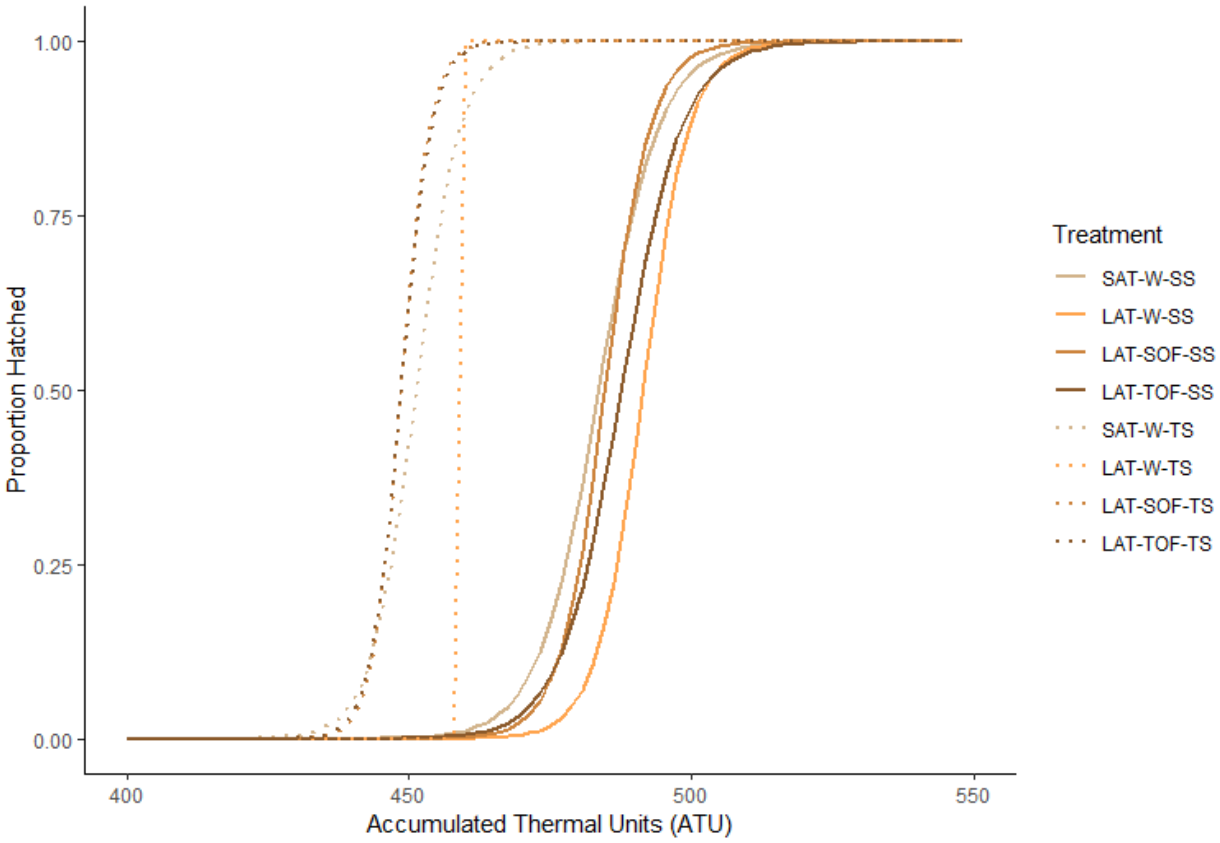
LAT-TOF-SS, n=160, -141.0346 Pr(>|z|) =<0.001, 1.411 Pr(>|z|) =<0.001

SAT-W-TS n=105, -154.025 Pr(>|z|) =<0.001, 1.683 Pr(>|z|) =<0.001

LAT-W-TS n=6, -114.598 Pr(>|z|) =<0.001, 1.232 Pr(>|z|) =<0.001

LAT-SOF-TS n=1, -2980.69 Pr(>|z|) =0.989, 31.54 Pr(>|z|) =0.989

LAT-TOF-TS n=13, -213.452 Pr(>|z|) =<0.001, 2.317 Pr(>|z|) =<0.001



Appendix Figure 3-B6: Logistic regressions of hatch timing for block 6 (solid lines =Salmon x Salmon crosses, dashed =Salmon x Trout crosses, equation=$y = \frac{1}{1+e^{-coefficient)*ATU-(intercept)}}$). Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid. Sample sizes, intercepts, coefficients, and corresponding Pr(>|z|) values for each treatment were as follows.

SAT-W-SS n=169, -87.672 Pr(>|z|) =<0.001, 0.901 Pr(>|z|) =<0.001

LAT-W-SS n=170, -121.177 Pr(>|z|) =<0.001, 1.225 Pr(>|z|) =<0.001

LAT-SOF-SS n=143, -122.611 Pr(>|z|) =<0.001, 1.257 Pr(>|z|) =<0.001

LAT-TOF-SS, n=160, -90.043 Pr(>|z|) =<0.001, 0.917 Pr(>|z|) =<0.001

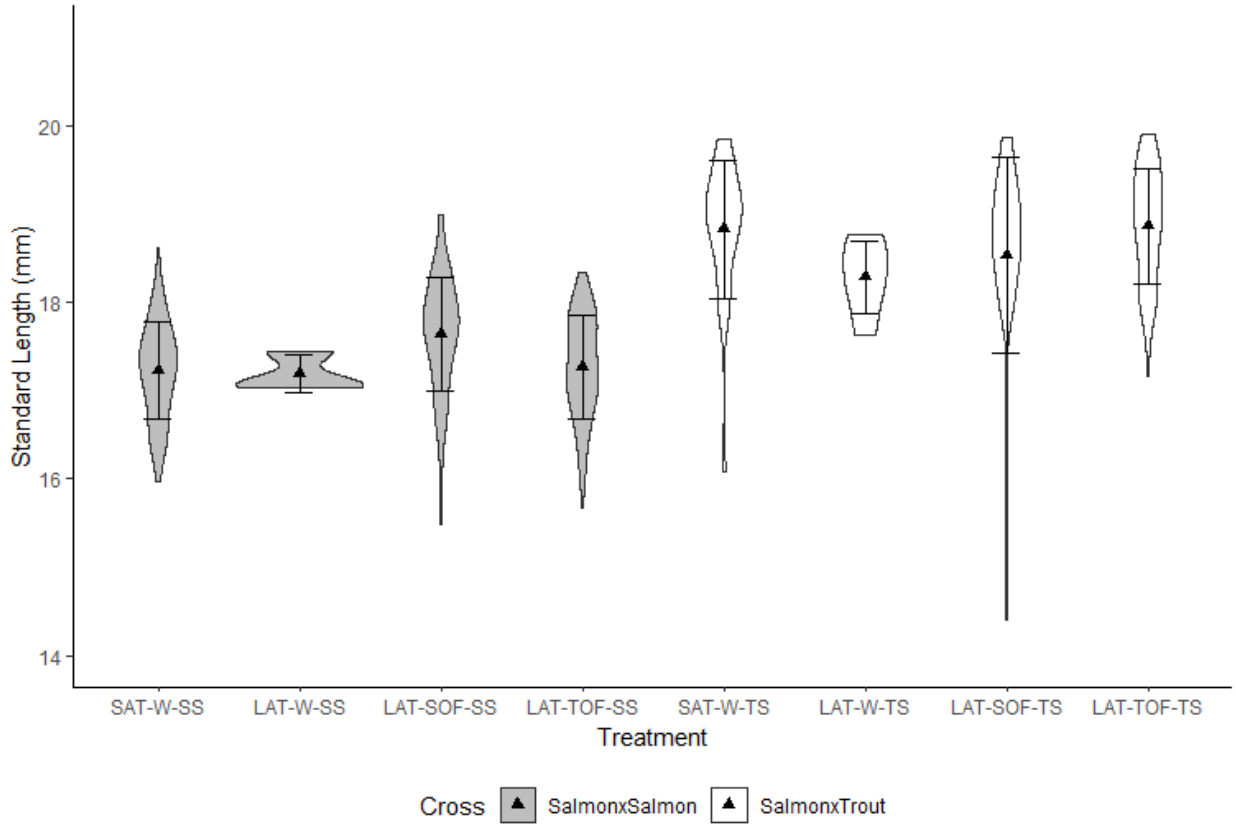
SAT-W-TS n=100, -116.555 Pr(>|z|) =<0.001, 1.281 Pr(>|z|) =<0.001

LAT-W-TS n=1, -2924.93 Pr(>|z|) =0.989, 31.62 Pr(>|z|) =0.989

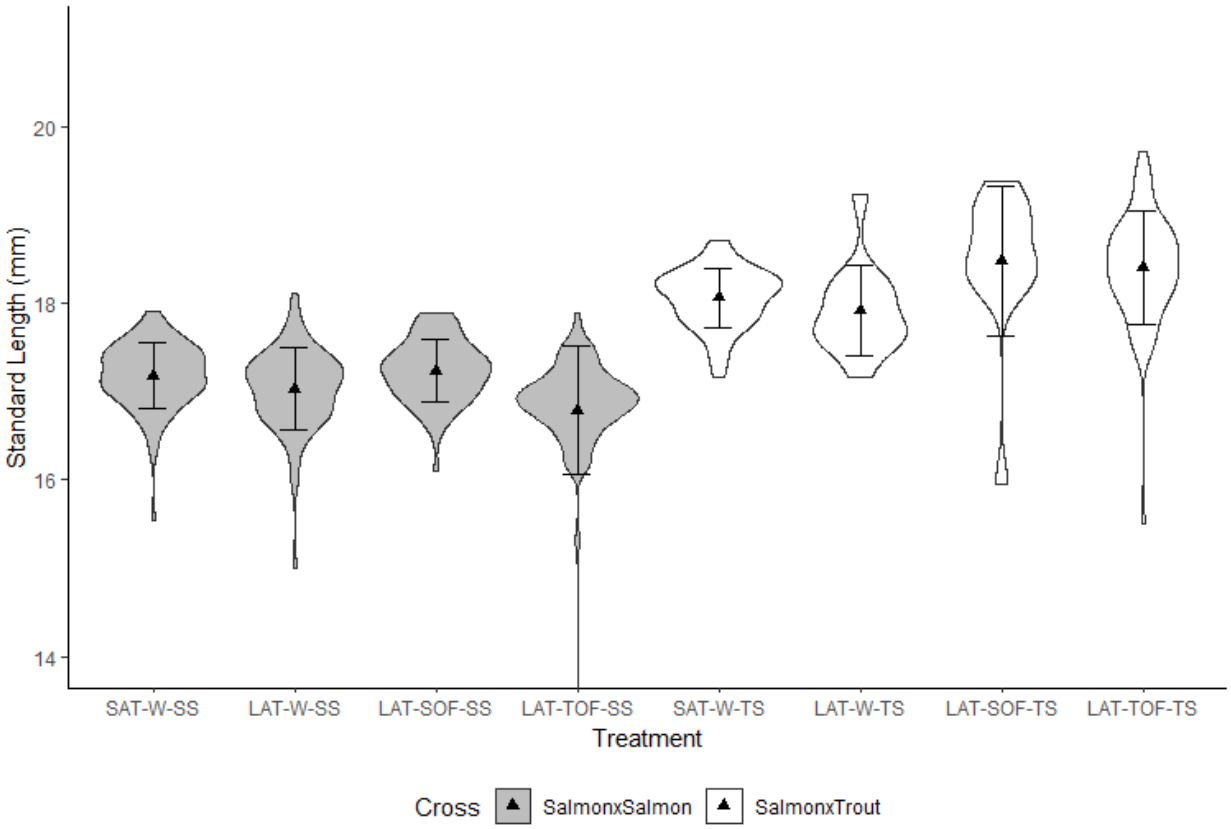
LAT-SOF-TS n=149, -187.044 Pr(>|z|) =<0.001, 2.067 Pr(>|z|) =<0.001

LAT-TOF-TS n=125, -174.468 Pr(>|z|) =<0.001, 1.928 Pr(>|z|) =<0.001

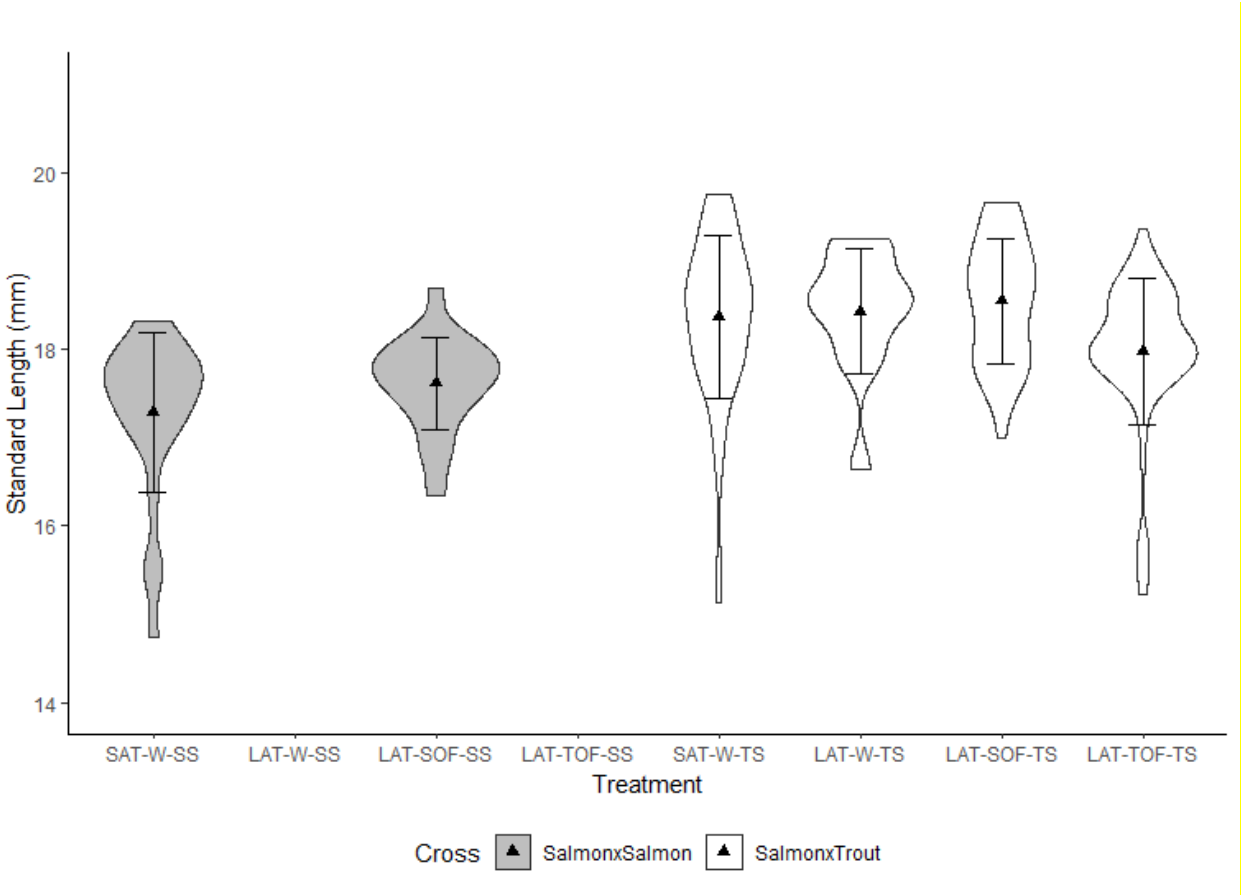
Appendix 3-C: Standard Length at hatch by block



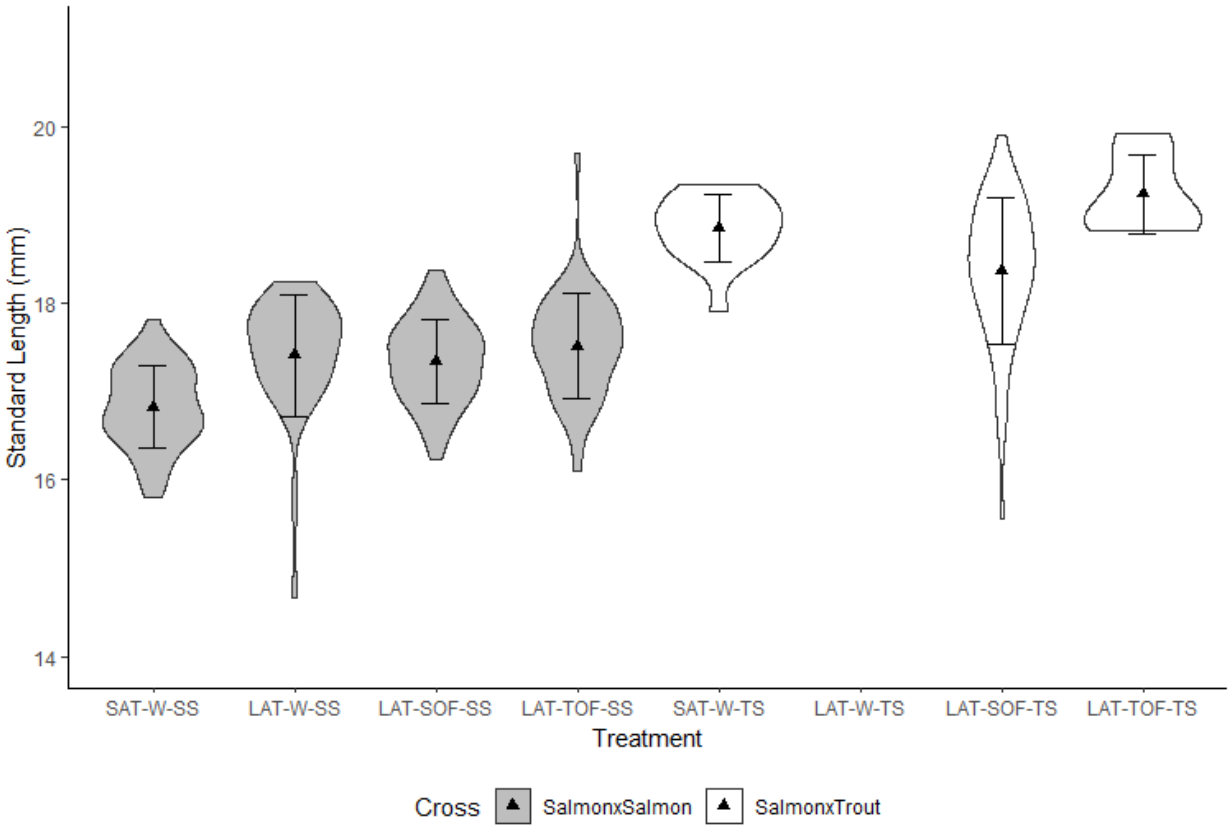
Appendix Figure 3-C1: Standard lengths of fish from block 1 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=73, LAT-W-SS n=3, LAT-SOF-SS n=88, LAT-TOF-SS n=78, SAT-W-TS n=26, LAT-W-TS n=8, LAT-SOF-TS n=44, LAT-TOF-TS n=52. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



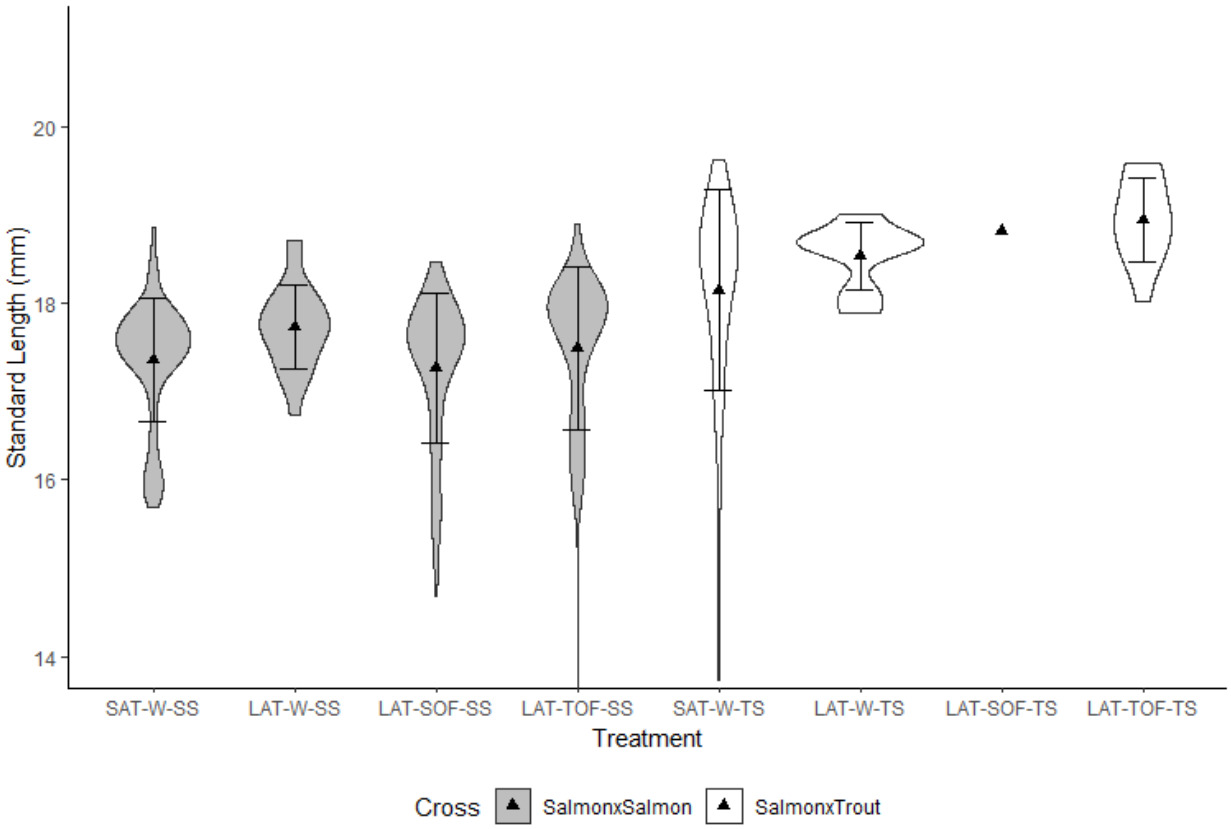
Appendix Figure 3-C2: Standard lengths of fish from block 2 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=87, LAT-W-SS n=68, LAT-SOF-SS n=69, LAT-TOF-SS n=81, SAT-W-TS n=36, LAT-W-TS n=13 LAT-SOF-TS n=13, LAT-TOF-TS n=58. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



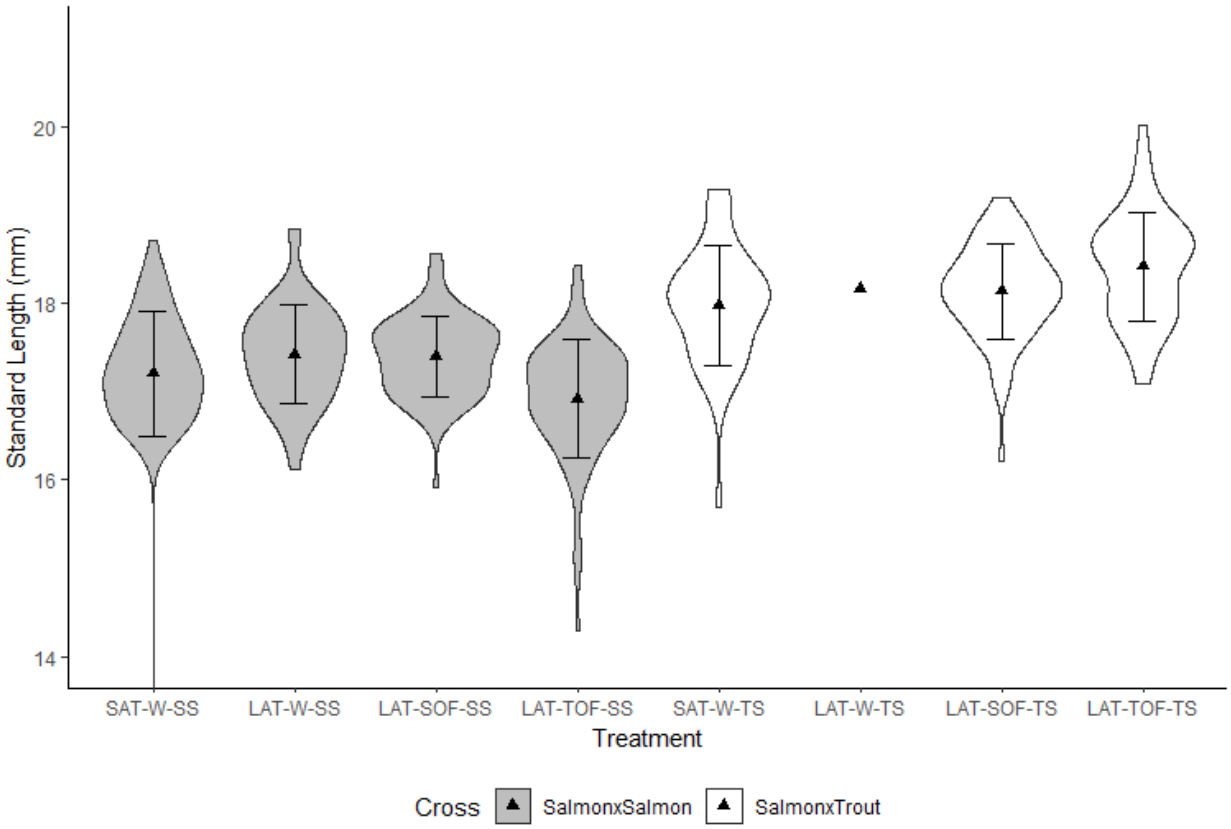
Appendix Figure 3-C3: Standard lengths of fish from block 3 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=24, LAT-W-SS n=0, LAT-SOF-SS n=68, LAT-TOF-SS n=0, SAT-W-TS n=59, LAT-W-TS n=12, LAT-SOF-TS n=31, LAT-TOF-TS n=69. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



Appendix Figure 3-C4: Standard lengths of fish from block 4 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=62, LAT-W-SS n=36, LAT-SOF-SS n=52, LAT-TOF-SS n=51, SAT-W-TS n=16, LAT-W-TS n=0 LAT-SOF-TS n=47, LAT-TOF-TS n=7. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

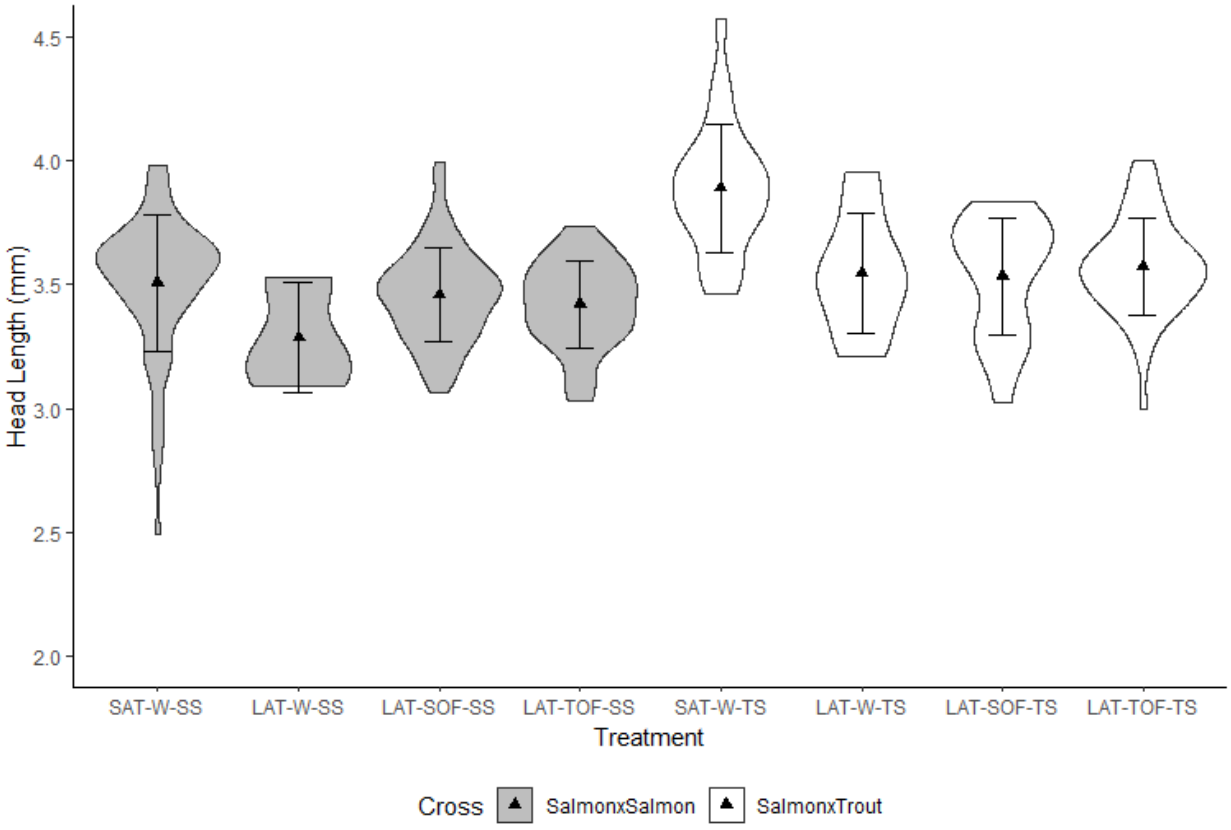


Appendix Figure 3-C5: Standard lengths of fish from block 5 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=65, LAT-W-SS n=22, LAT-SOF-SS n=79, LAT-TOF-SS n=87, SAT-W-TS n=62, LAT-W-TS n=7 LAT-SOF-TS n=1, LAT-TOF-TS n=13. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

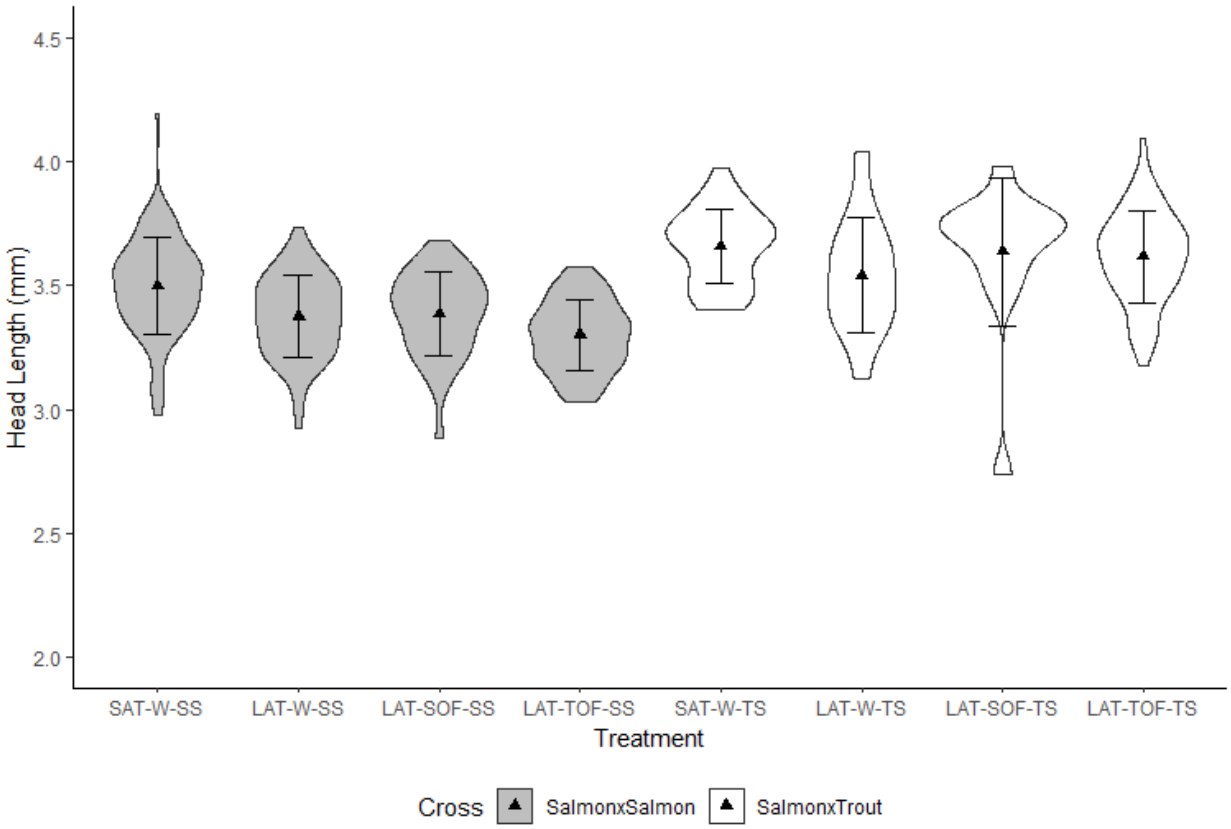


Appendix Figure 3-C6: Standard lengths of fish from block 6 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data Sample sizes for each treatment were as follows; SAT-W-SS n=90, LAT-W-SS n=47, LAT-SOF-SS n=82, LAT-TOF-SS n=84, SAT-W-TS n=45, LAT-W-TS n=1 LAT-SOF-TS n=66, LAT-TOF-TS n=55. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

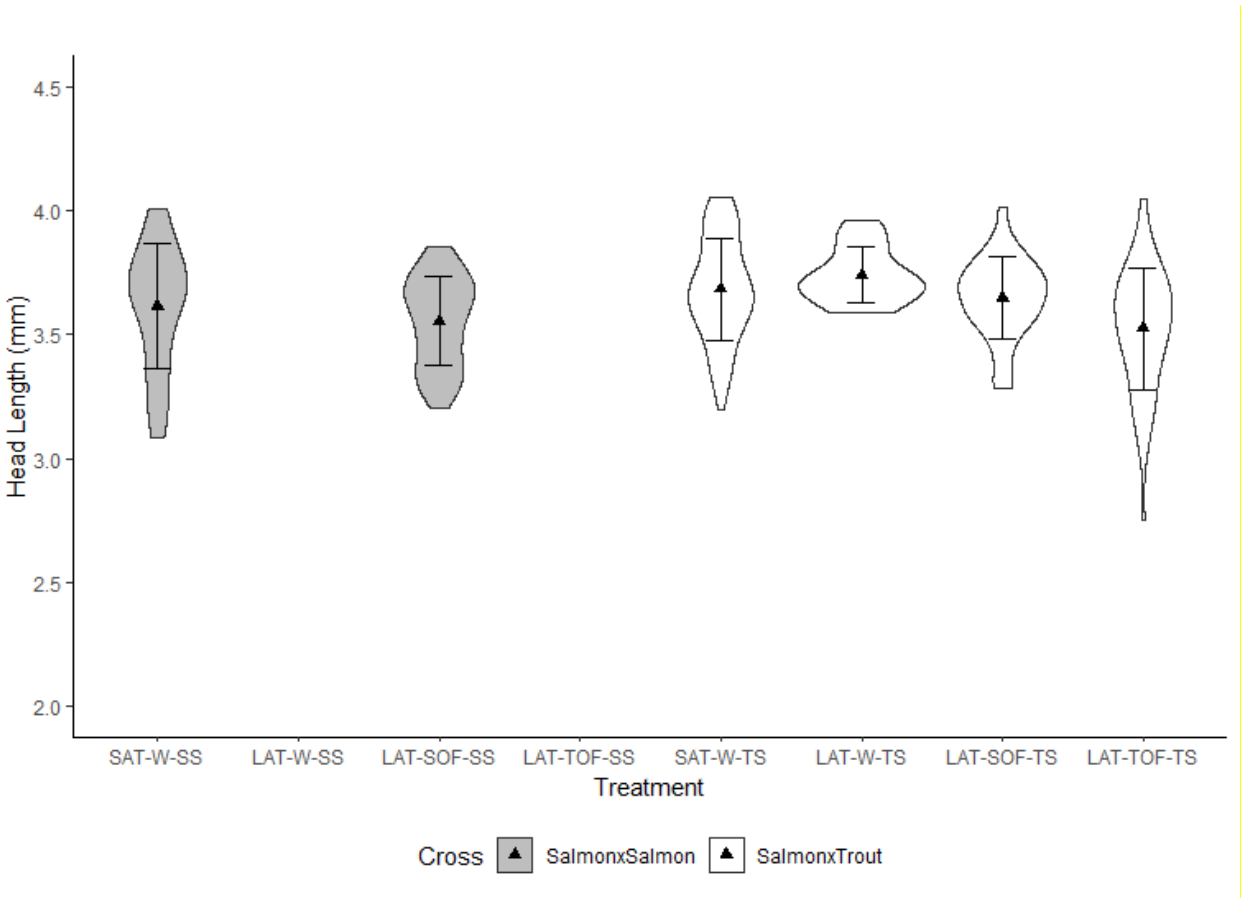
Appendix 3-D: Head length at hatch by block



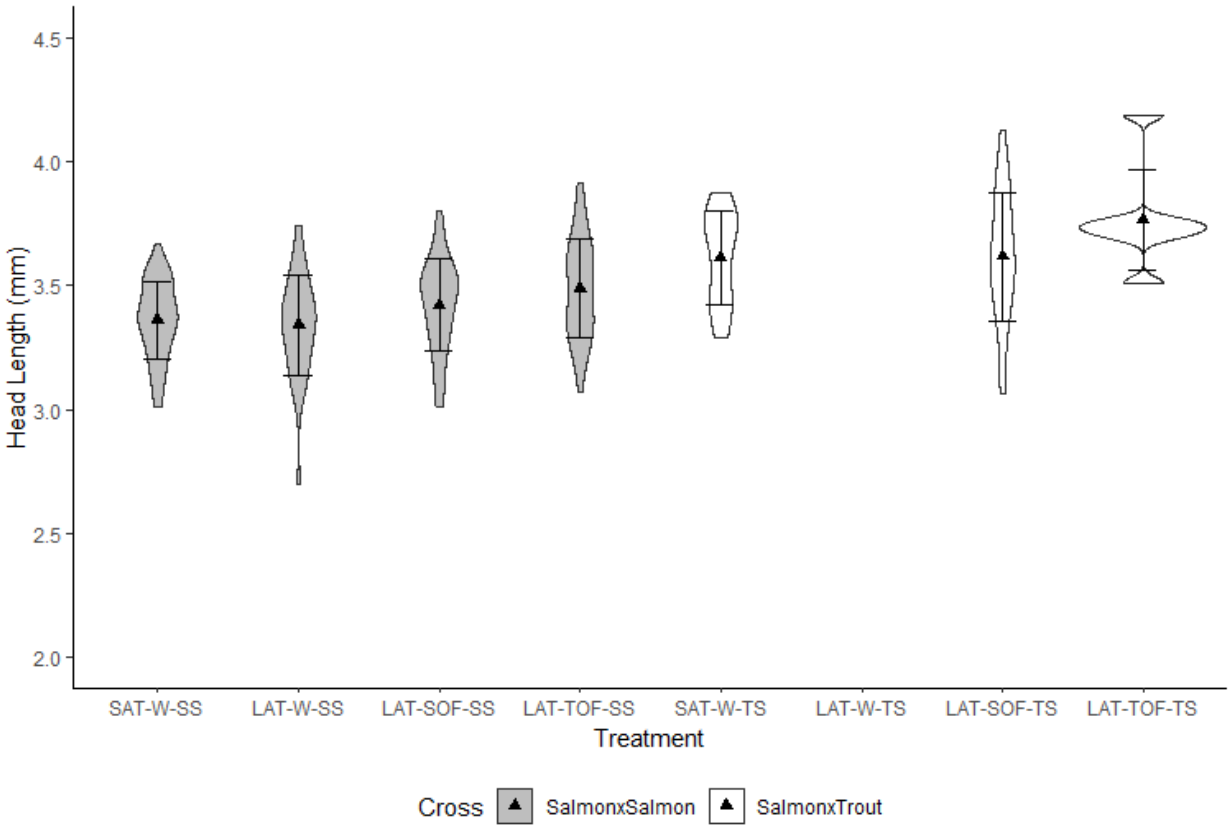
Appendix Figure 3-D1: The head lengths of fish from block 1 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=73, LAT-W-SS n=3, LAT-SOF-SS n=88, LAT-TOF-SS n=79, SAT-W-TS n=25, LAT-W-TS n=8, LAT-SOF-TS n=44, LAT-TOF-TS n=52. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



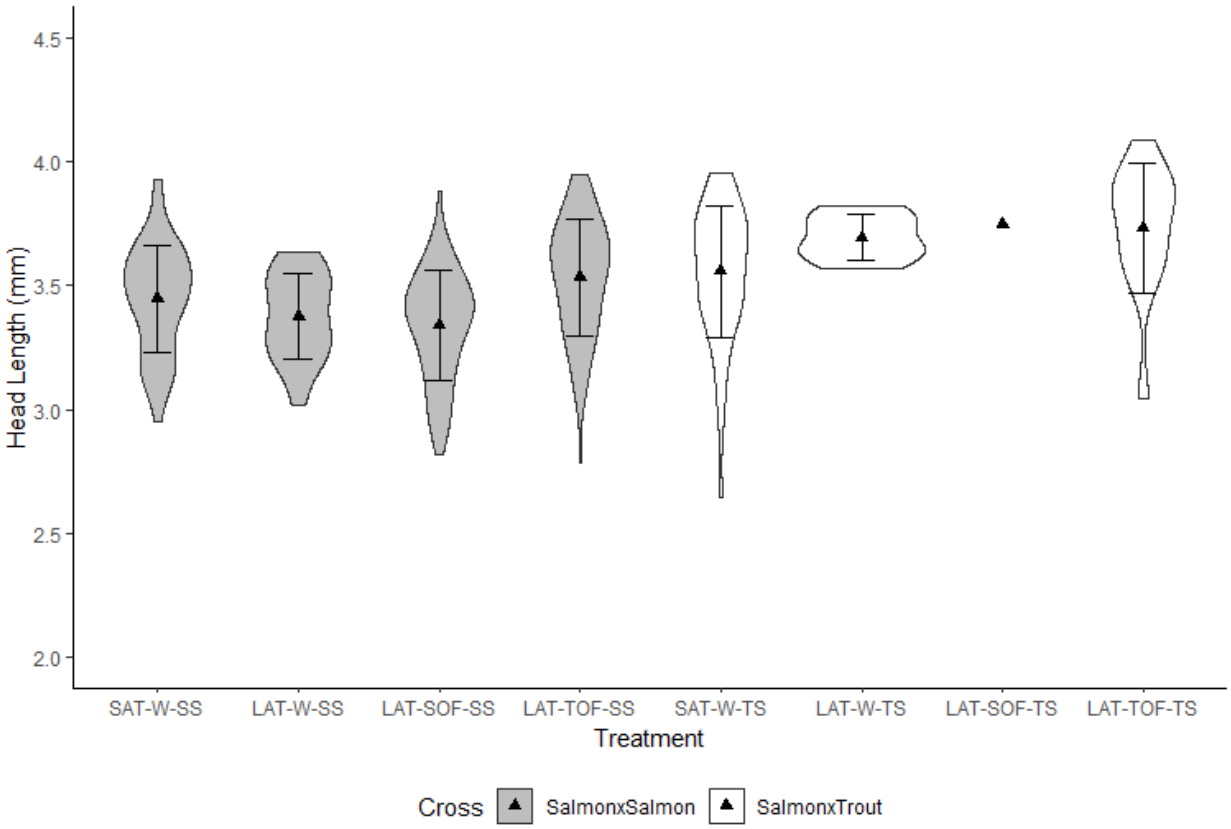
Appendix Figure 3-D2: Head lengths of fish from block 2 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=87, LAT-W-SS n=68, LAT-SOF-SS n=72, LAT-TOF-SS n=80, SAT-W-TS n=36, LAT-W-TS n=14, LAT-SOF-TS n=13, LAT-TOF-TS n=59. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



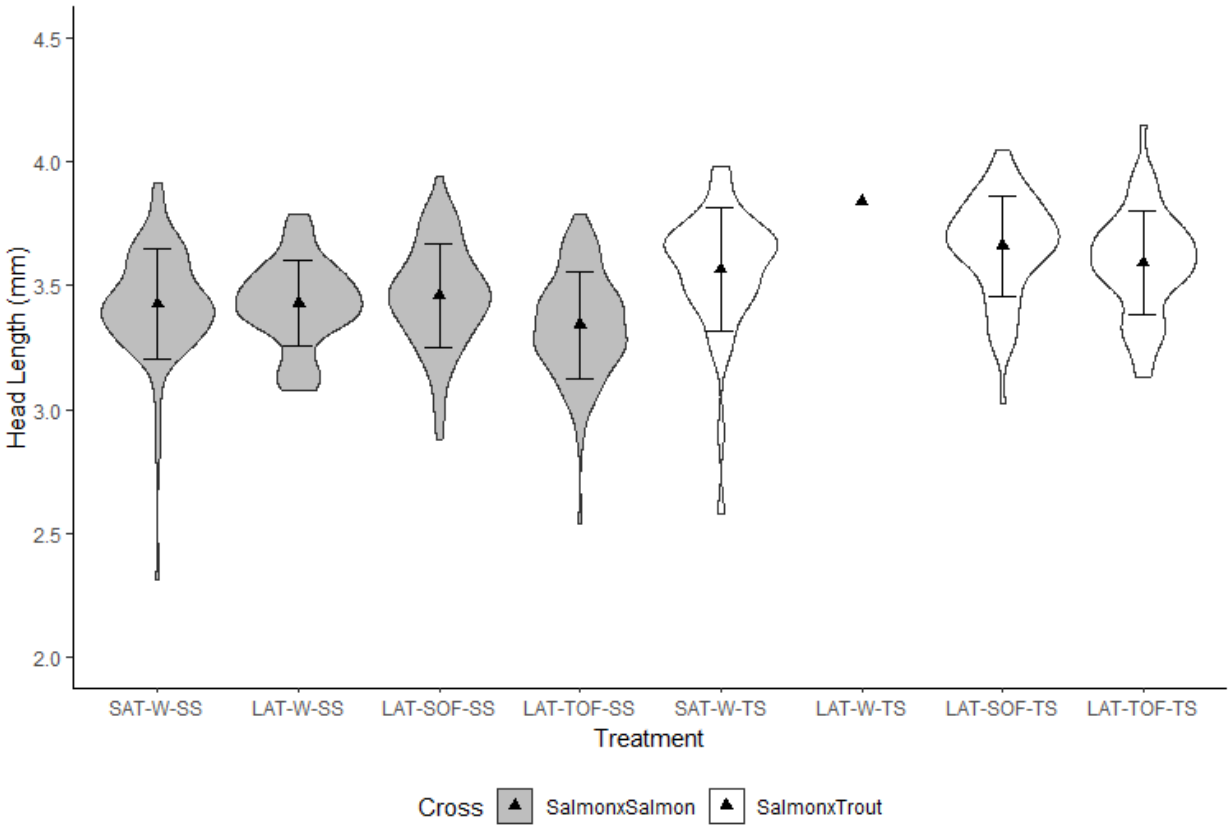
Appendix Figure 3-D3: Head lengths of fish from block 3 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=26, LAT-W-SS n=0, LAT-SOF-SS n=68, LAT-TOF-SS n=0, SAT-W-TS n=60, LAT-W-TS n=12, LAT-SOF-TS n=32, LAT-TOF-TS n=69. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



Appendix Figure 3-D4: Head lengths of fish from block 4 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=62, LAT-W-SS n=37, LAT-SOF-SS n=52, LAT-TOF-SS n=53, SAT-W-TS n=17, LAT-W-TS n=0, LAT-SOF-TS n=46, LAT-TOF-TS n=7. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

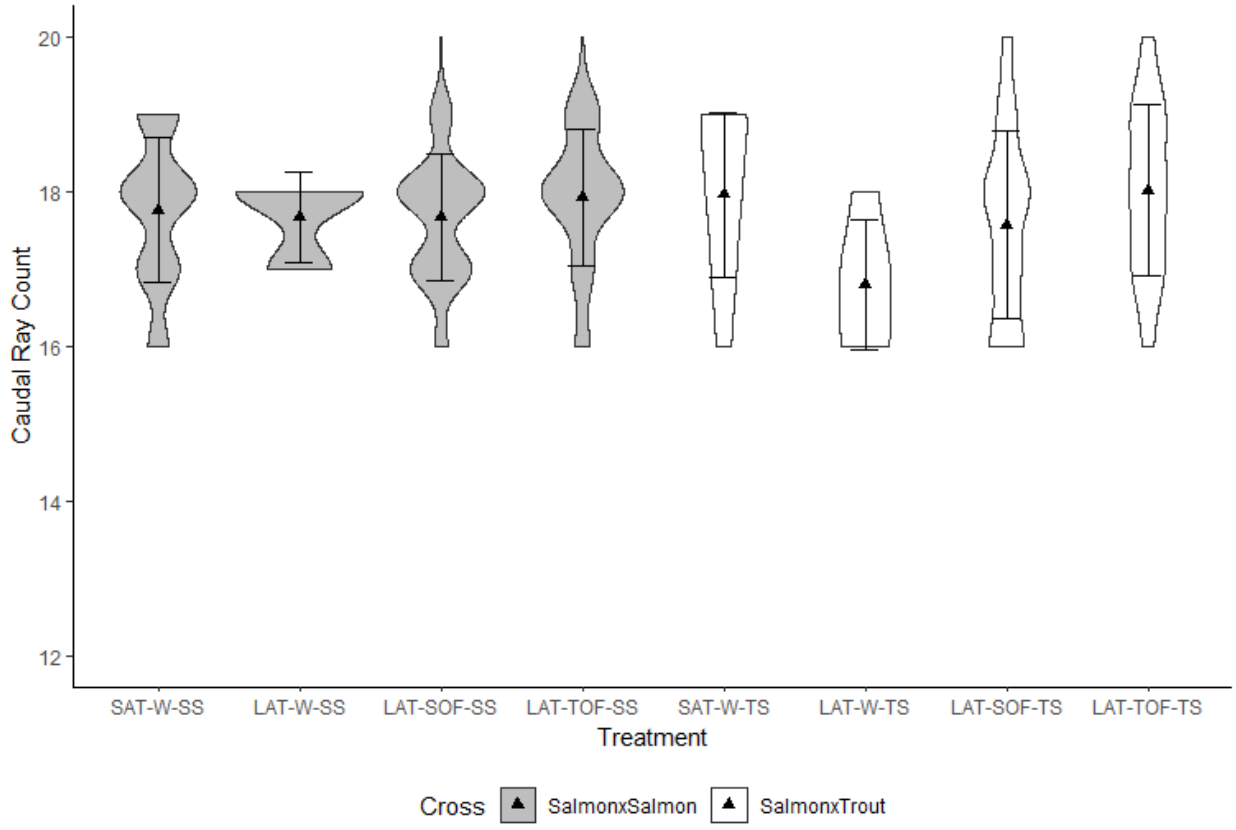


Appendix Figure 3-D5: Head lengths of fish from block 5 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=62, LAT-W-SS n=20, LAT-SOF-SS n=78, LAT-TOF-SS n=84, SAT-W-TS n=51, LAT-W-TS n=7, LAT-SOF-TS n=1, LAT-TOF-TS n=13. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

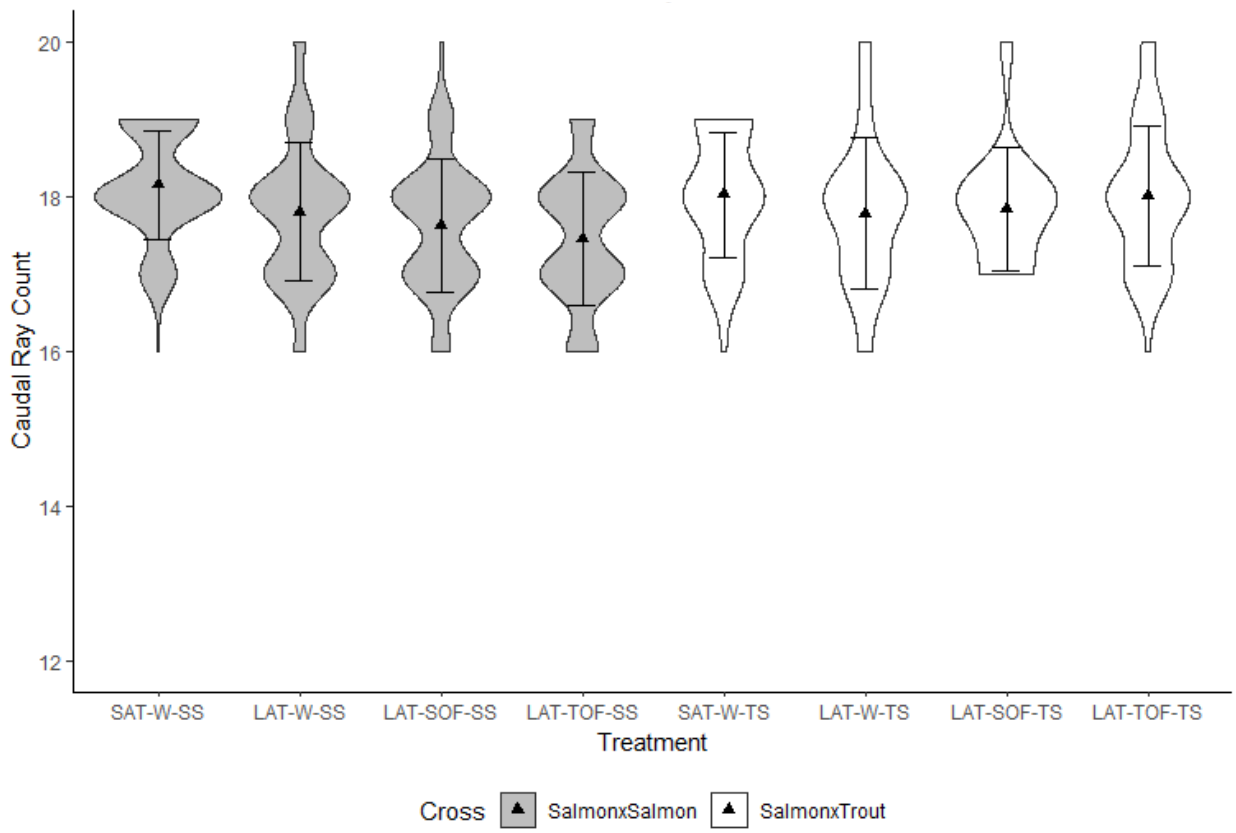


Appendix Figure 3-D6: Head lengths of fish from block 6 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=90, LAT-W-SS n=47, LAT-SOF-SS n=82, LAT-TOF-SS n=85, SAT-W-TS n=46, LAT-W-TS n=1, LAT-SOF-TS n=66, LAT-TOF-TS n=55. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

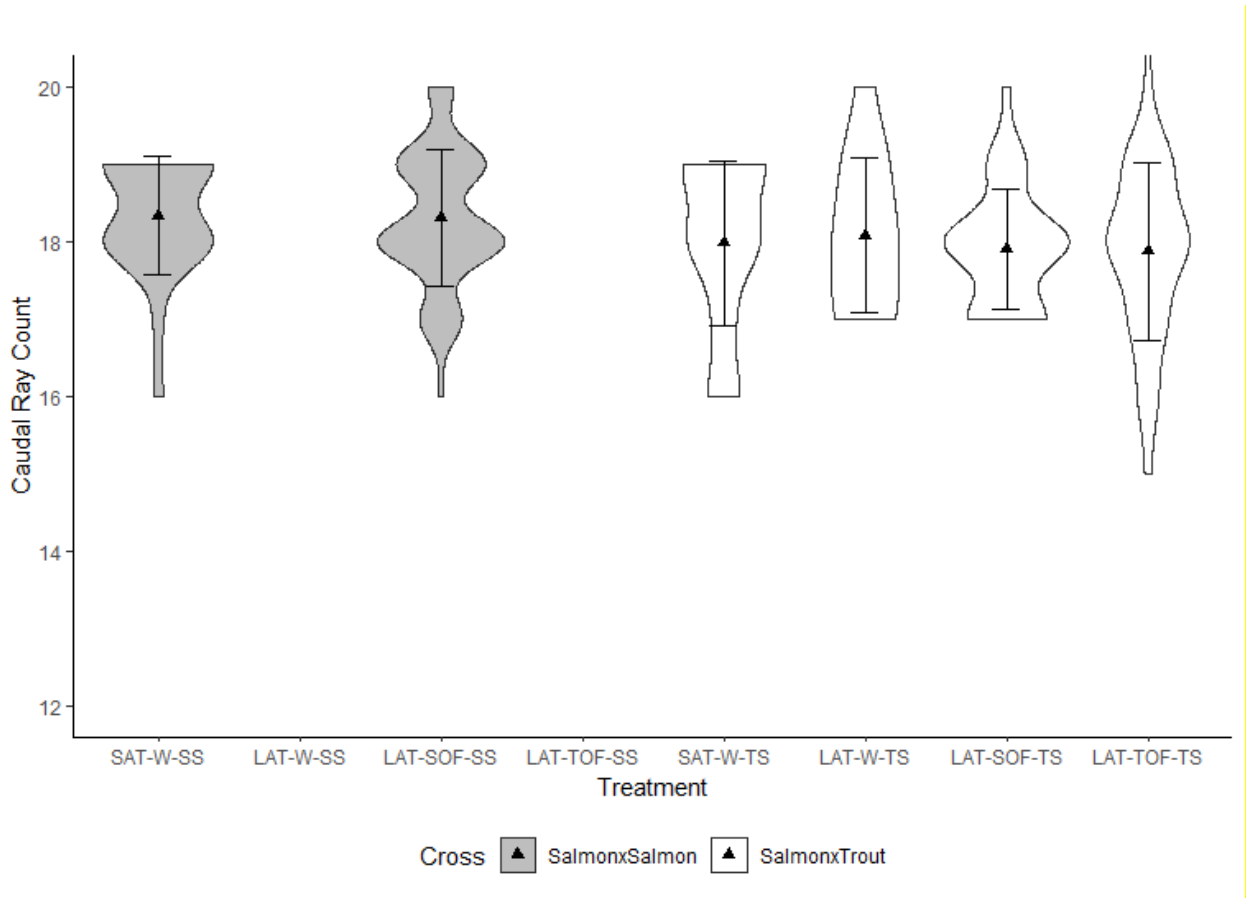
Appendix 3-E: Caudal ray count at hatch by block



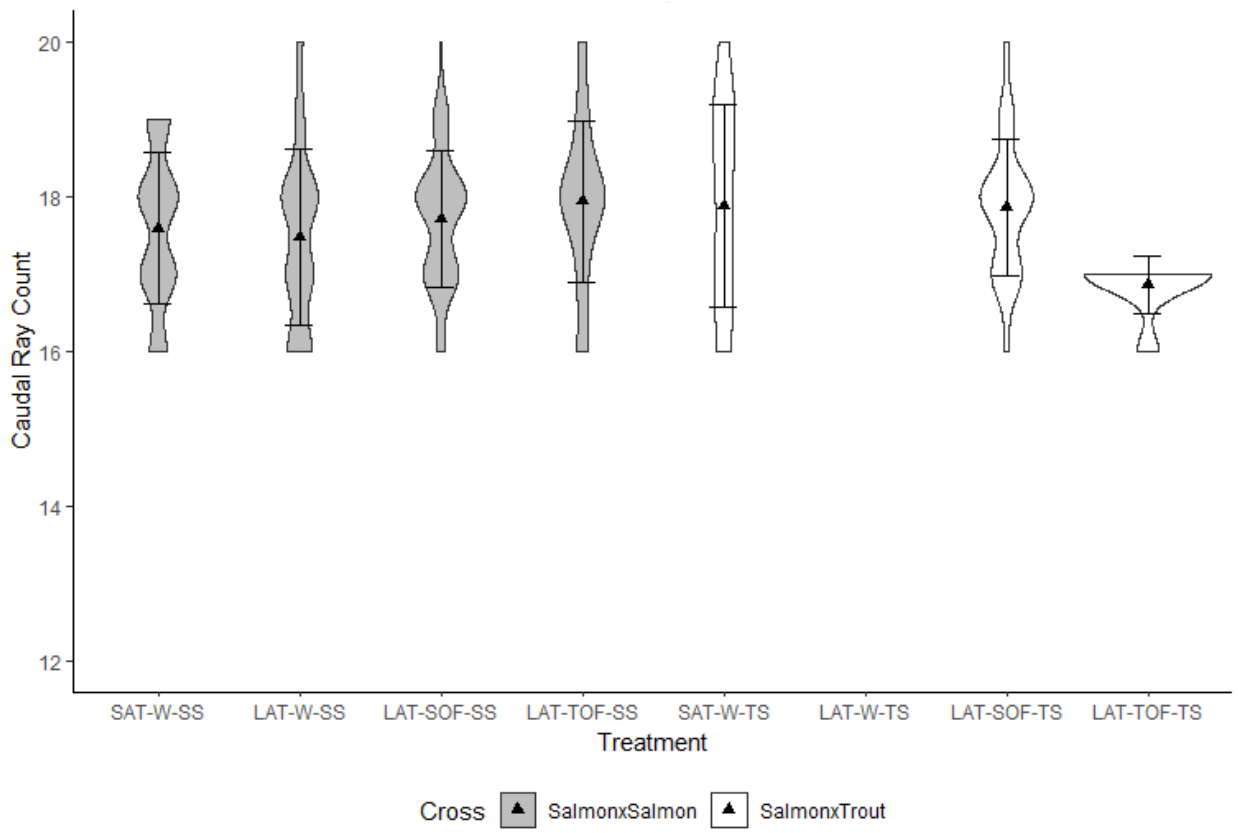
Appendix Figure 3-E1: Caudal fin ray counts of fish from block 1 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=71, LAT-W-SS n=3, LAT-SOF-SS n=85, LAT-TOF-SS n=77, SAT-W-TS n=25, LAT-W-TS n=6, LAT-SOF-TS n=44, LAT-TOF-TS n=49. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



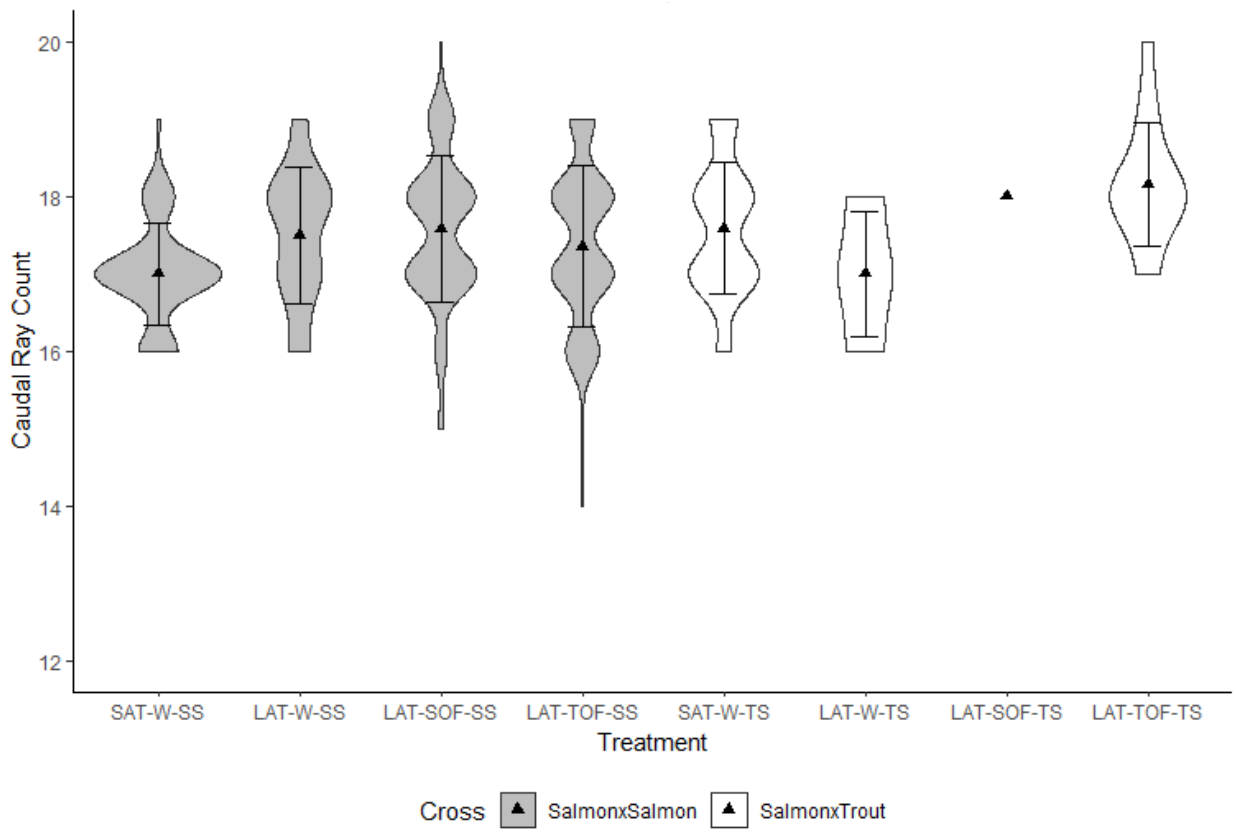
Appendix Figure 3-E2: Caudal fin ray counts of fish from block 2 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=87, LAT-W-SS n=64, LAT-SOF-SS n=70, LAT-TOF-SS n=78, SAT-W-TS n=36, LAT-W-TS n=14, LAT-SOF-TS n=13, LAT-TOF-TS n=56. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



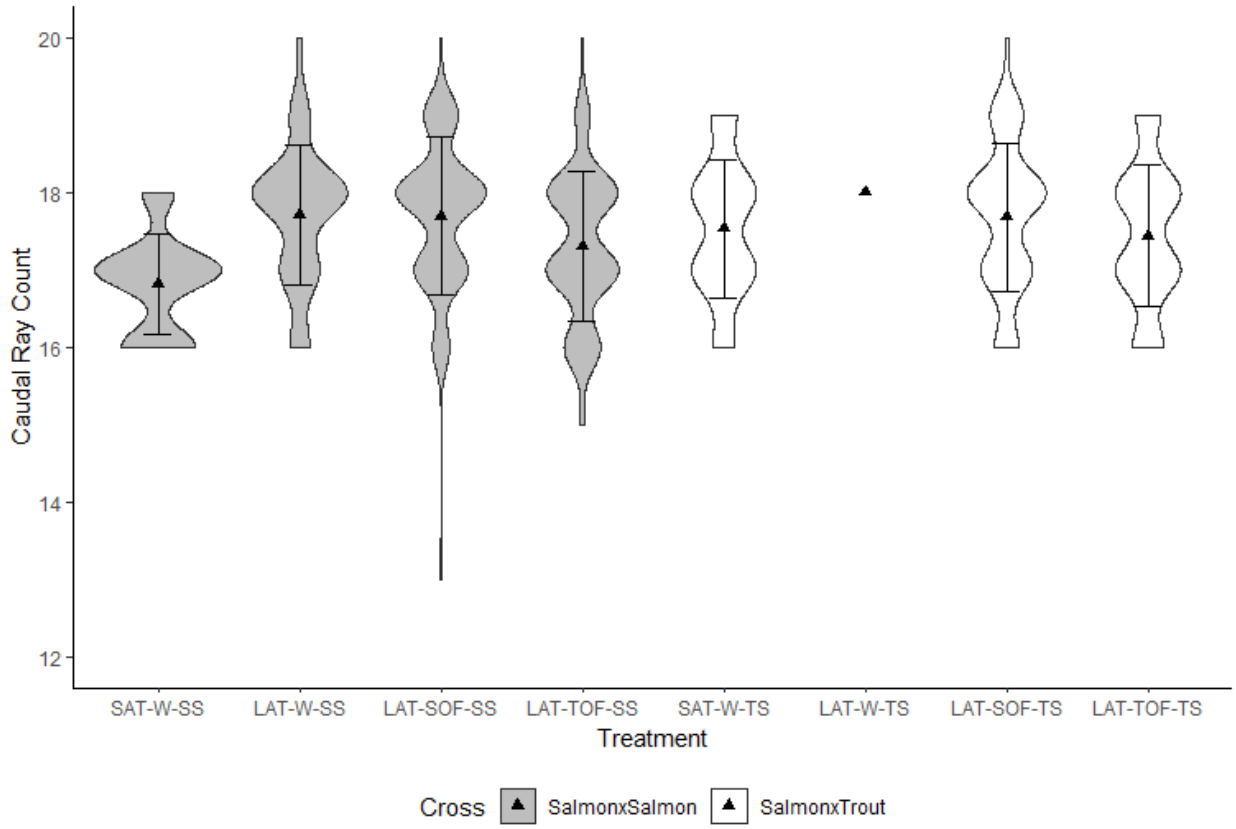
Appendix Figure 3-E3: Caudal fin ray counts of fish from block 3 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=24, LAT-W-SS n=0, LAT-SOF-SS n=68, LAT-TOF-SS n=0, SAT-W-TS n=58, LAT-W-TS n=12, LAT-SOF-TS n=32, LAT-TOF-TS n=71. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



Appendix Figure 3-E4: Caudal fin ray counts of fish from block 4 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=61, LAT-W-SS n=34, LAT-SOF-SS n=51, LAT-TOF-SS n=50, SAT-W-TS n=16, LAT-W-TS n=0, LAT-SOF-TS n=43, LAT-TOF-TS n=7. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

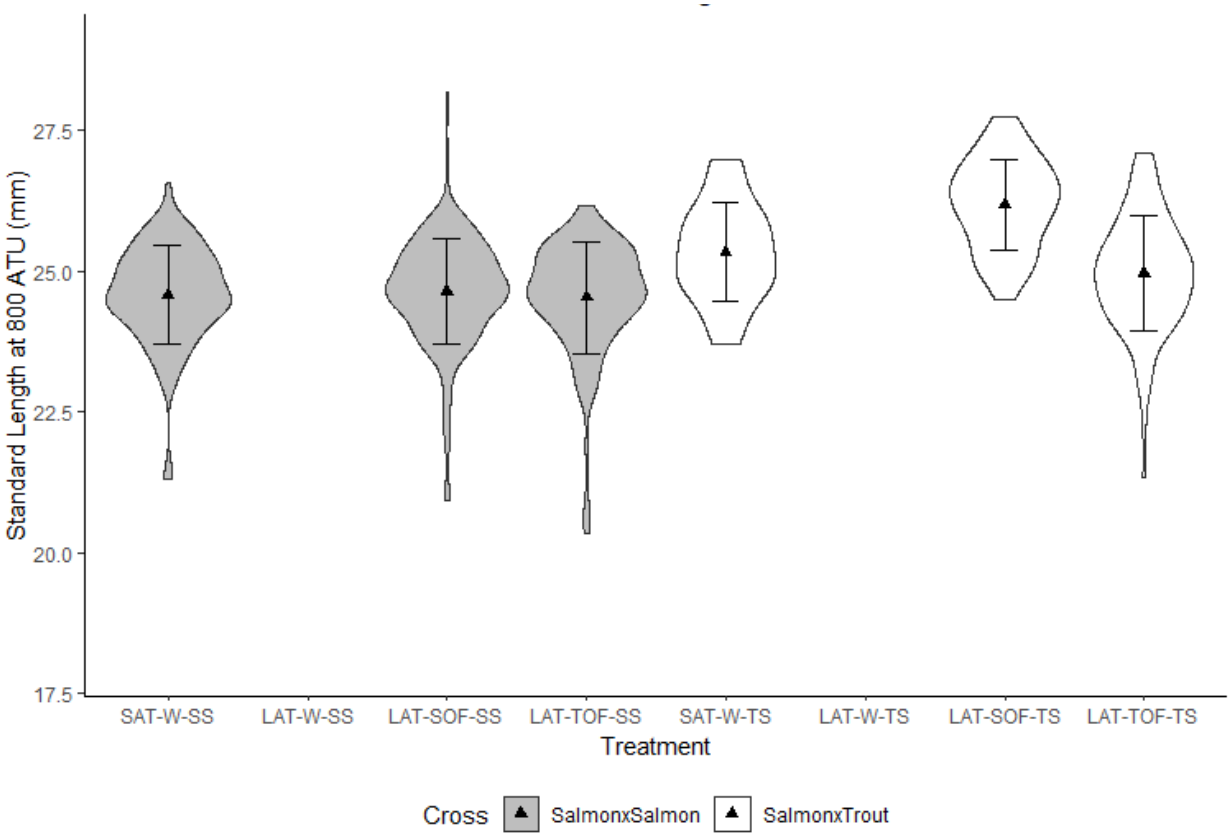


Appendix Figure 3-E5: Caudal fin ray counts of fish from block 5 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=62, LAT-W-SS n=20, LAT-SOF-SS n=78, LAT-TOF-SS n=84, SAT-W-TS n=51, LAT-W-TS n=7, LAT-SOF-TS n=1, LAT-TOF-TS n=13. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

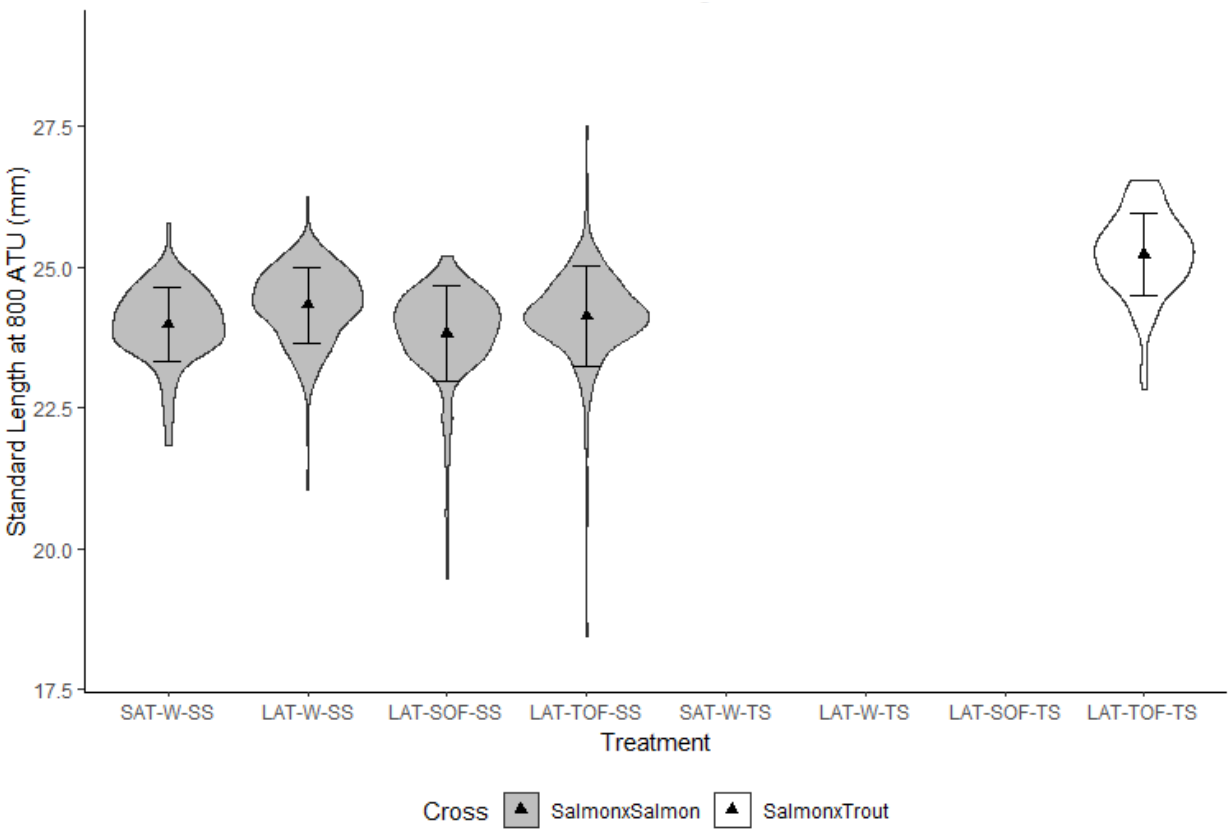


Appendix Figure 3-E6: Caudal fin ray counts of fish from block 6 at hatch. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=88, LAT-W-SS n=45, LAT-SOF-SS n=82, LAT-TOF-SS n=83, SAT-W-TS n=41, LAT-W-TS n=14 LAT-SOF-TS n=63, LAT-TOF-TS n=54. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

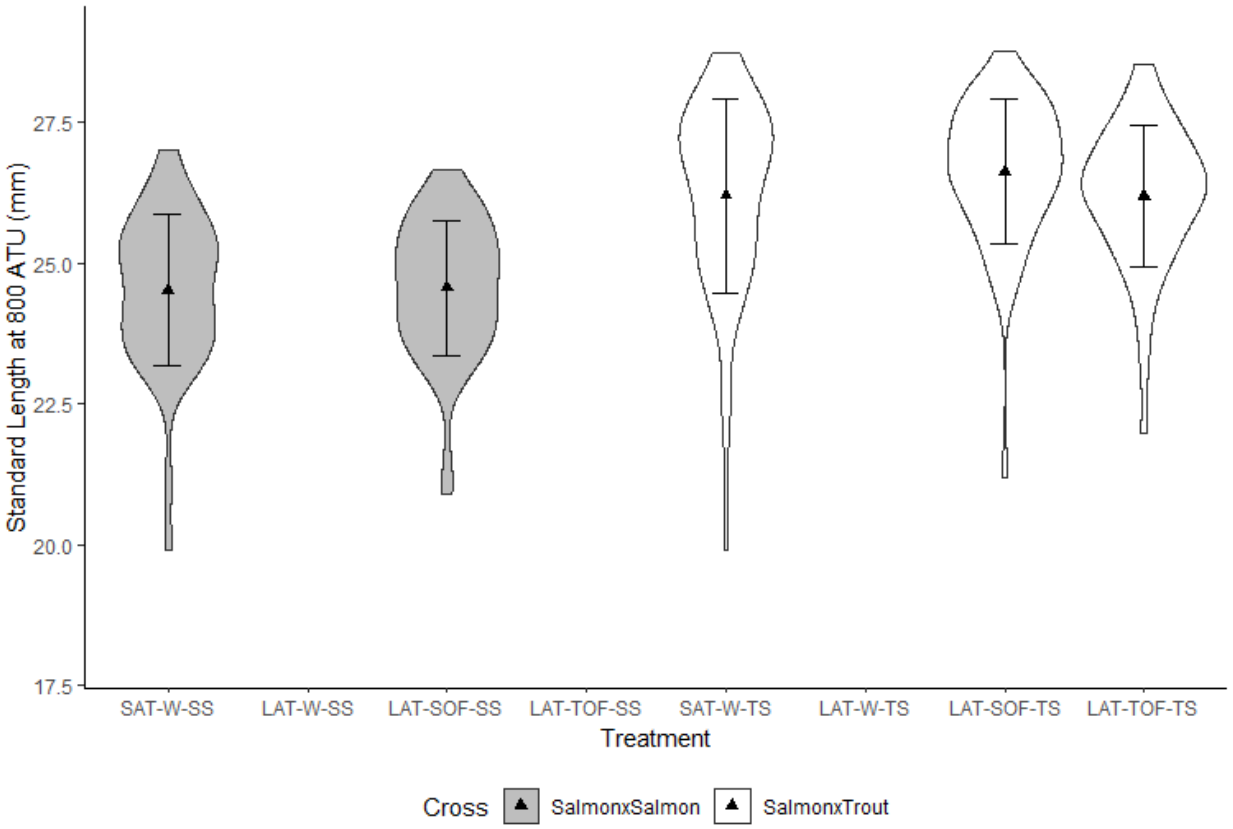
Appendix Figure 3-F: Standard Length at 800 ATU by block



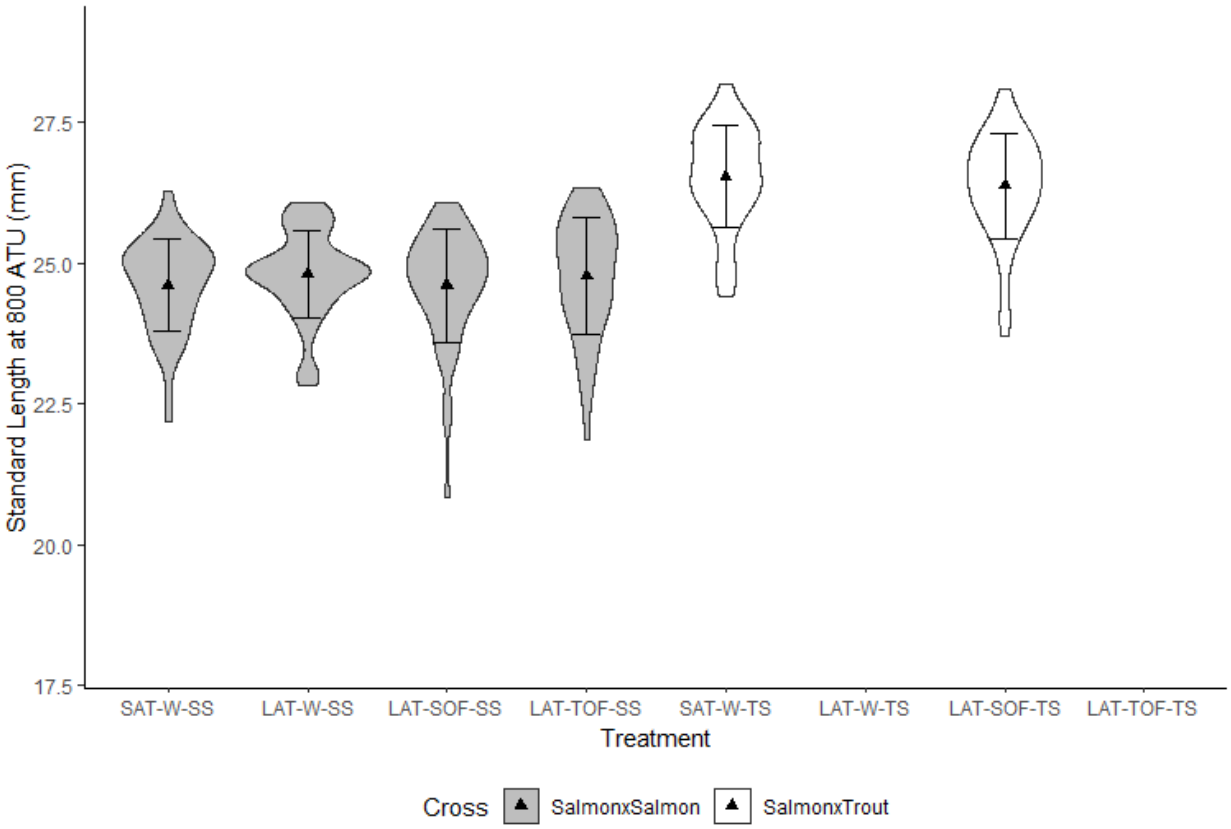
Appendix Figure 3-F1: Standard lengths of fish from block 1 at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=133, LAT-W-SS n=0, LAT-SOF-SS n=171, LAT-TOF-SS n=130, SAT-W-TS n=37, LAT-W-TS n=0 LAT-SOF-TS n=36, LAT-TOF-TS n=104. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



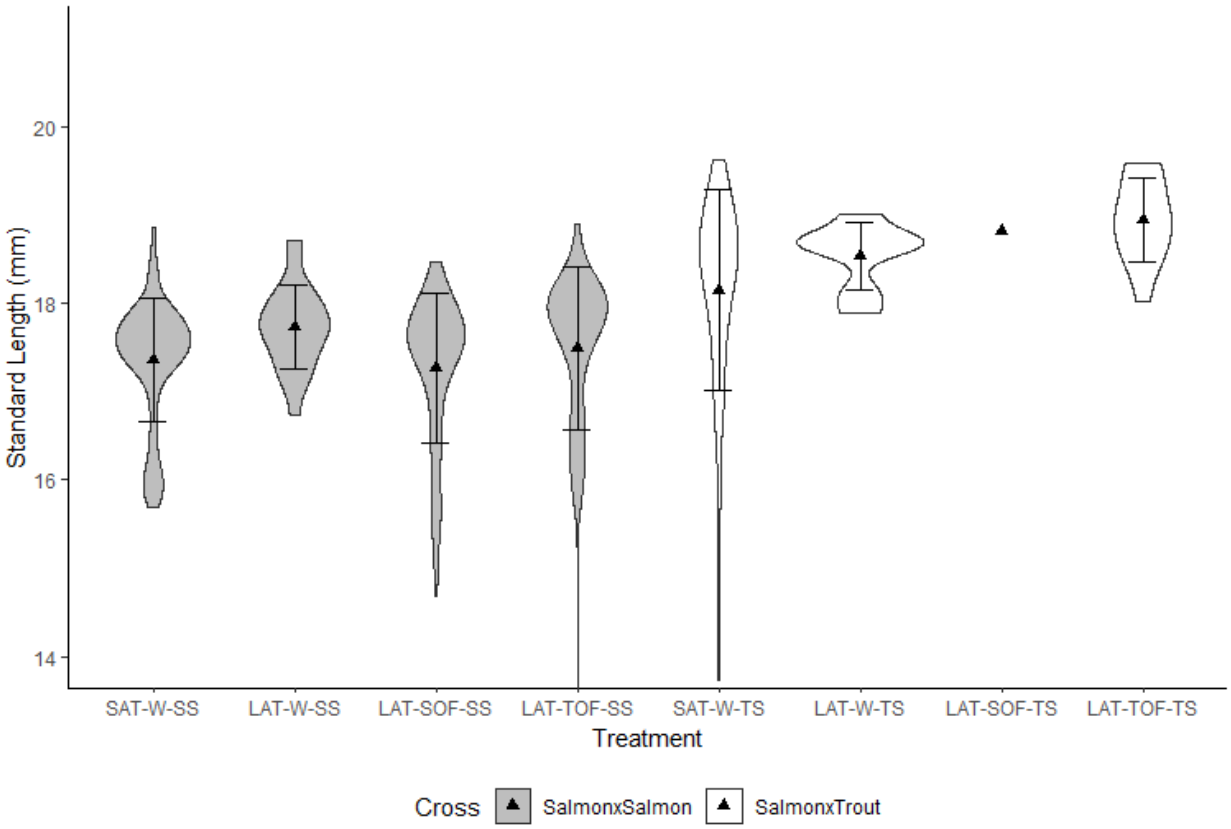
Appendix Figure 3-F2: Standard lengths of fish from block 2 at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=118, LAT-W-SS n=144, LAT-SOF-SS n=134, LAT-TOF-SS n=115, SAT-W-TS n=0, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=115. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



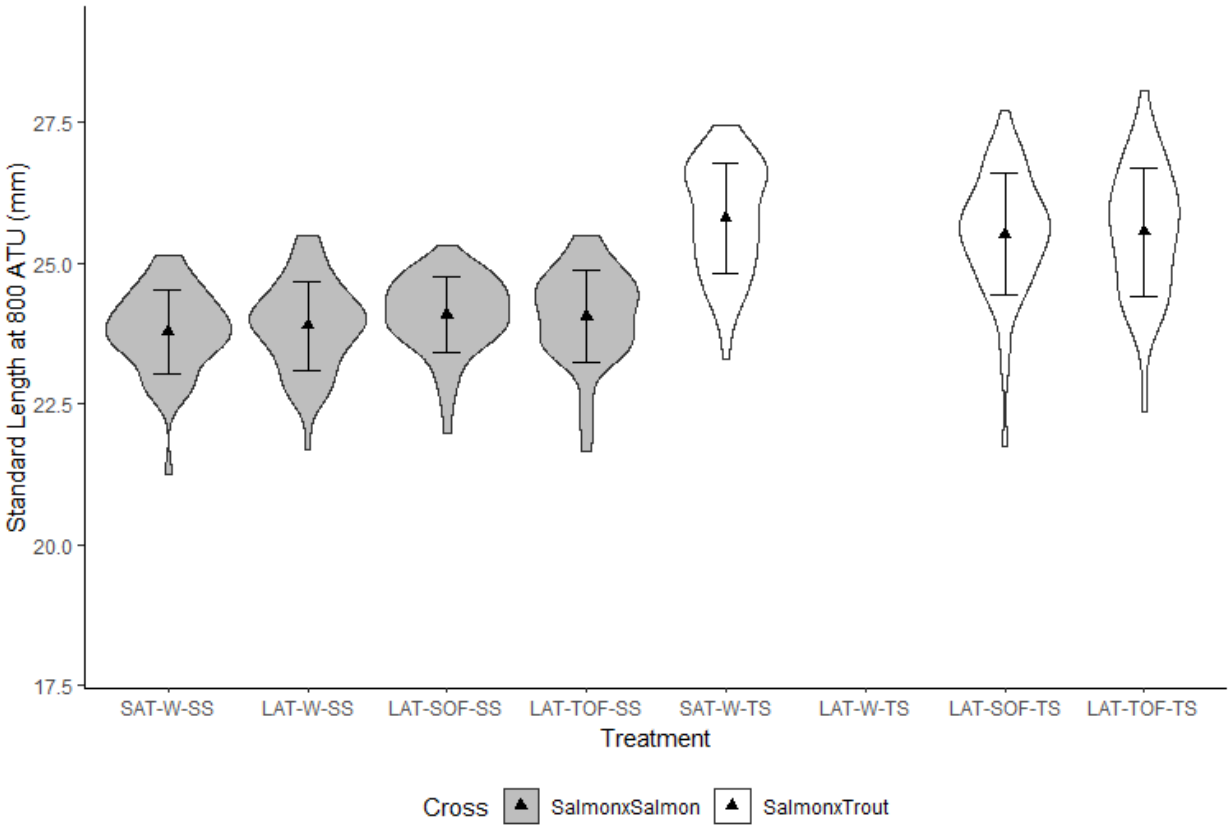
Appendix Figure 3-E3: Standard lengths of fish from block 3 at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=49, LAT-W-SS n=0, LAT-SOF-SS n=49, LAT-TOF-SS n=0, SAT-W-TS n=49, LAT-W-TS n=0 LAT-SOF-TS n=49, LAT-TOF-TS n=50. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



Appendix Figure 3-F4: Standard lengths of fish from block 4 at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=50, LAT-SOF-SS n=50, LAT-TOF-SS n=50, SAT-W-TS n=37, LAT-W-TS n=0 LAT-SOF-TS n=50, LAT-TOF-TS n=0. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

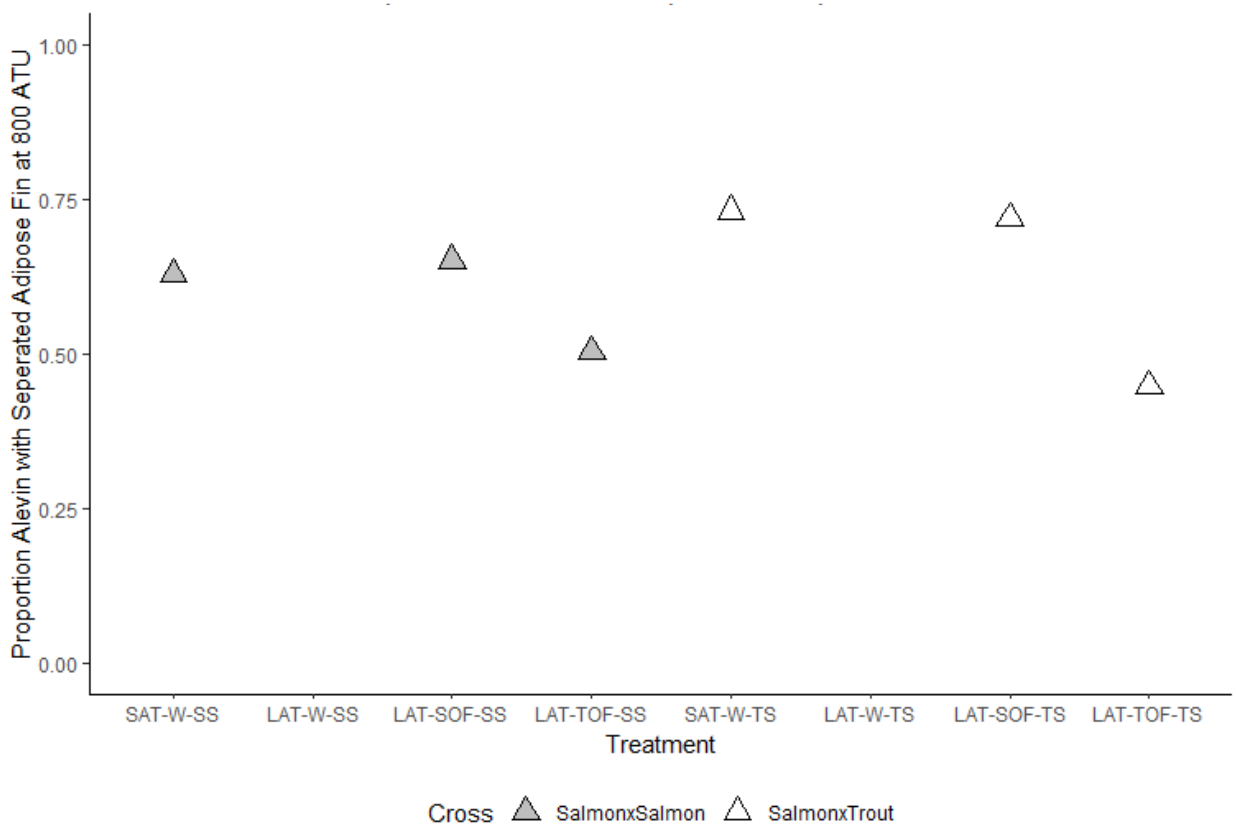


Appendix Figure 3-E5: Standard lengths of fish from block 5 at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=0, LAT-SOF-SS n=50, LAT-TOF-SS n=50, SAT-W-TS n=50, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=0. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

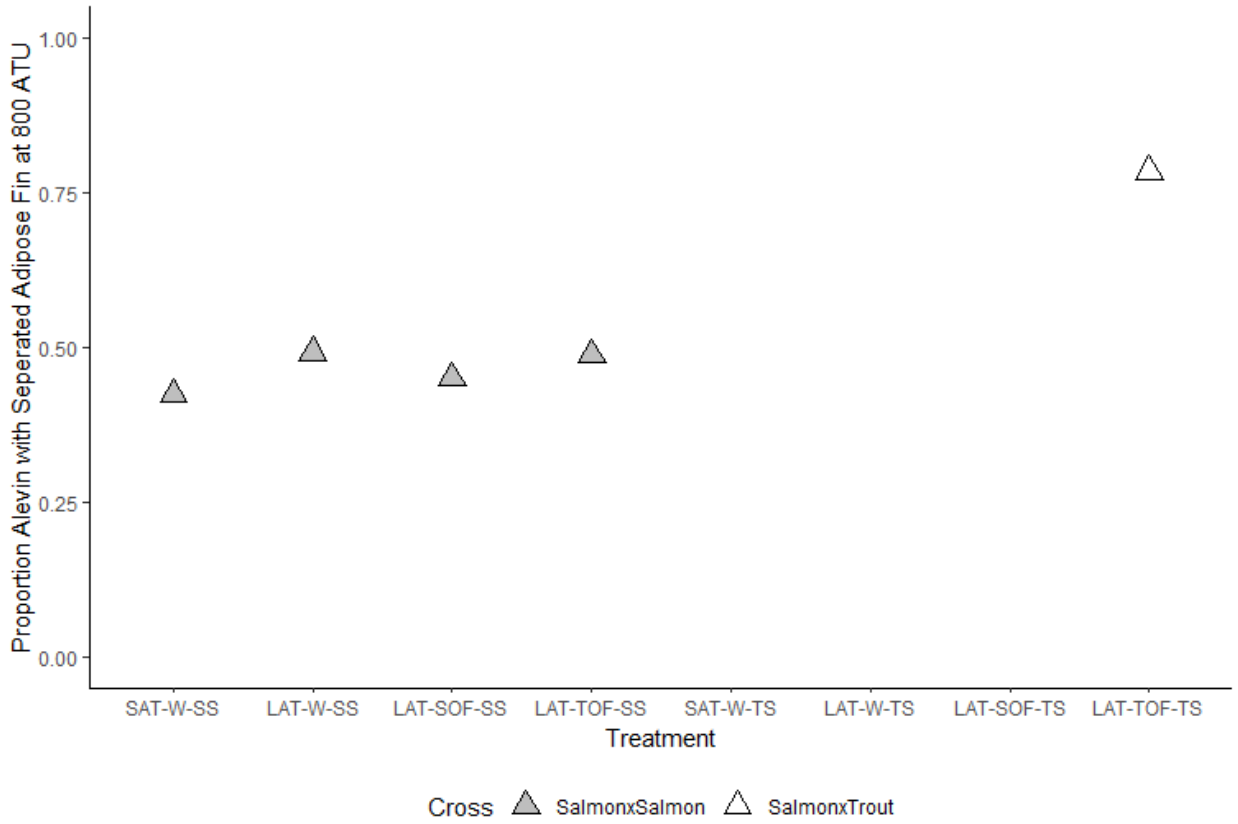


Appendix Figure 3-E6: Standard lengths of fish from block 6 at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=50, LAT-SOF-SS n=50, LAT-TOF-SS n=50, SAT-W-TS n=50, LAT-W-TS n=0 LAT-SOF-TS n=50, LAT-TOF-TS n=49. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

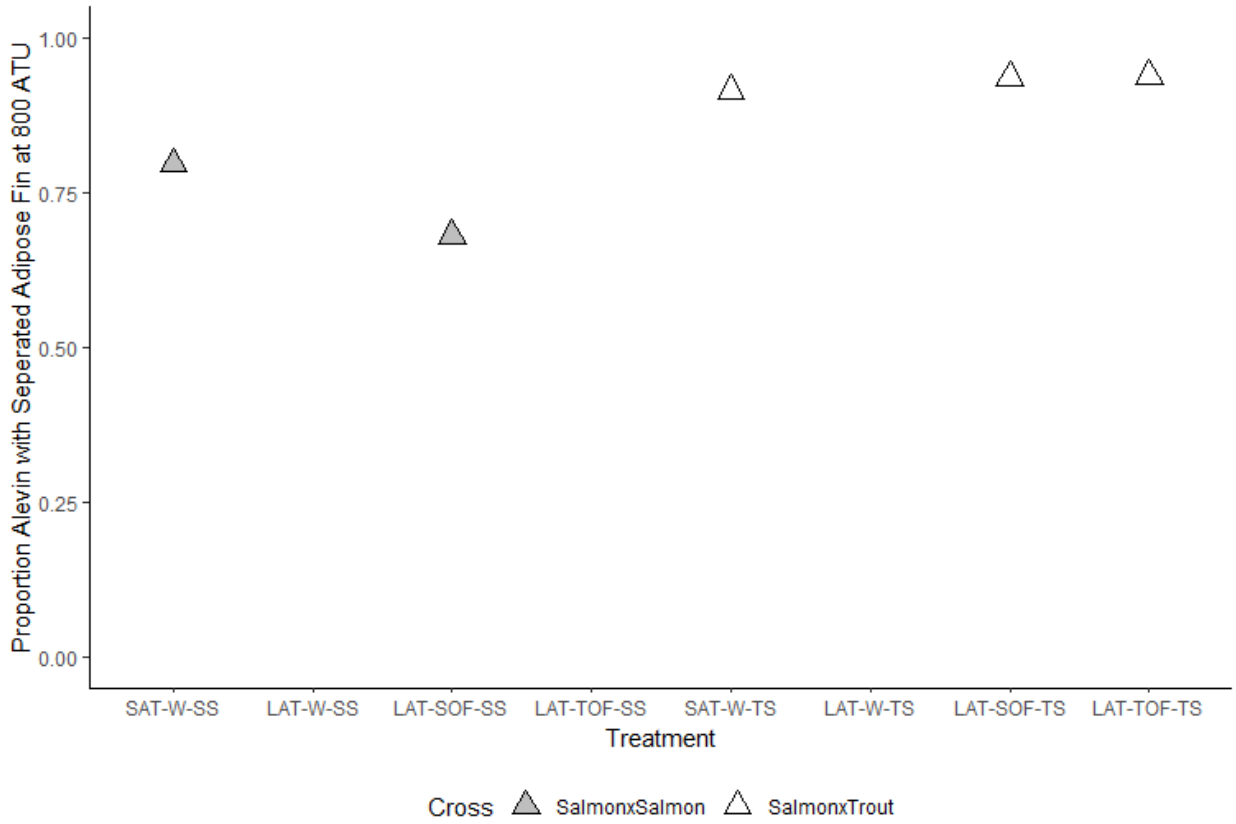
Appendix 3-G: Proportion of alevin with separated adipose fin by block



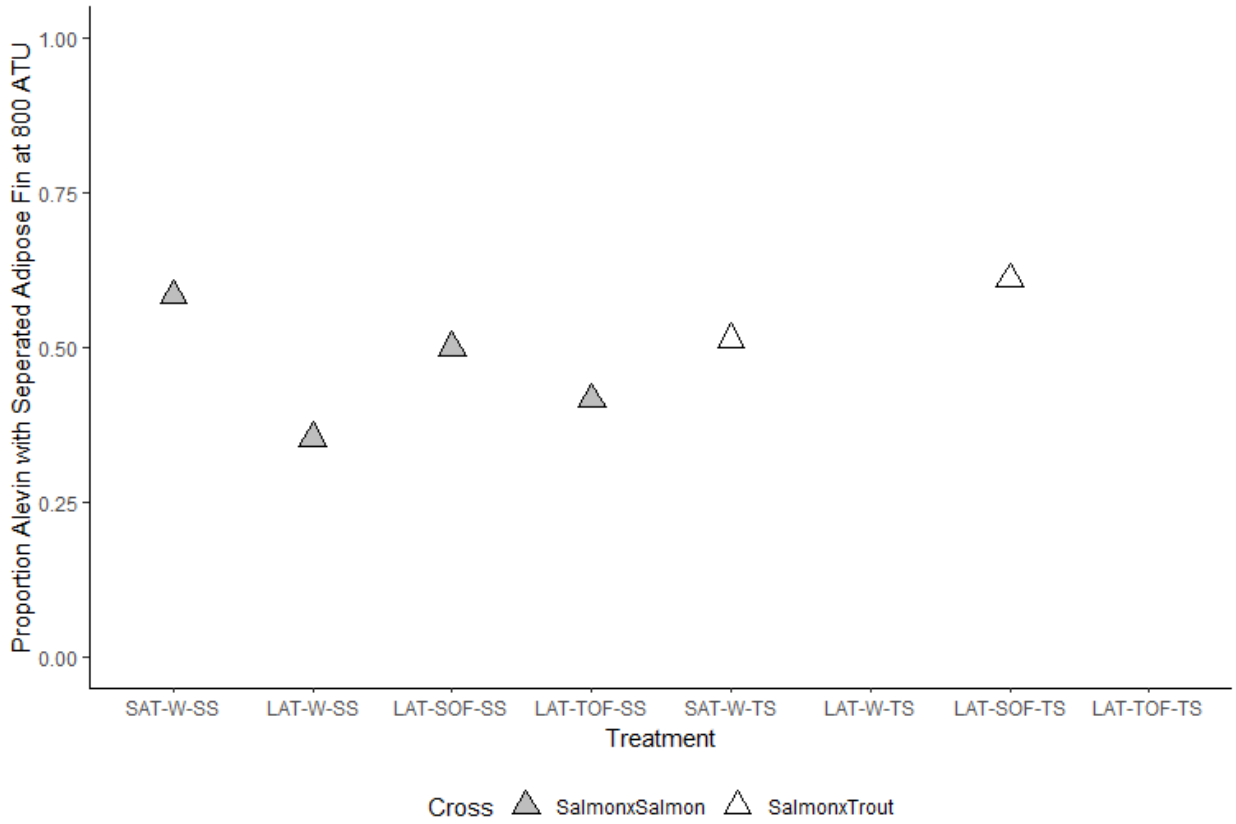
Appendix Figure 3-G1: Proportion of block 1 fish with separated adipose fins at 800 ATU. Triangles represent the means for each treatment. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=132, LAT-W-SS n=0, LAT-SOF-SS n=155, LAT-TOF-SS n=105, SAT-W-TS n=41, LAT-W-TS n=0 LAT-SOF-TS n=32, LAT-TOF-TS n=105. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



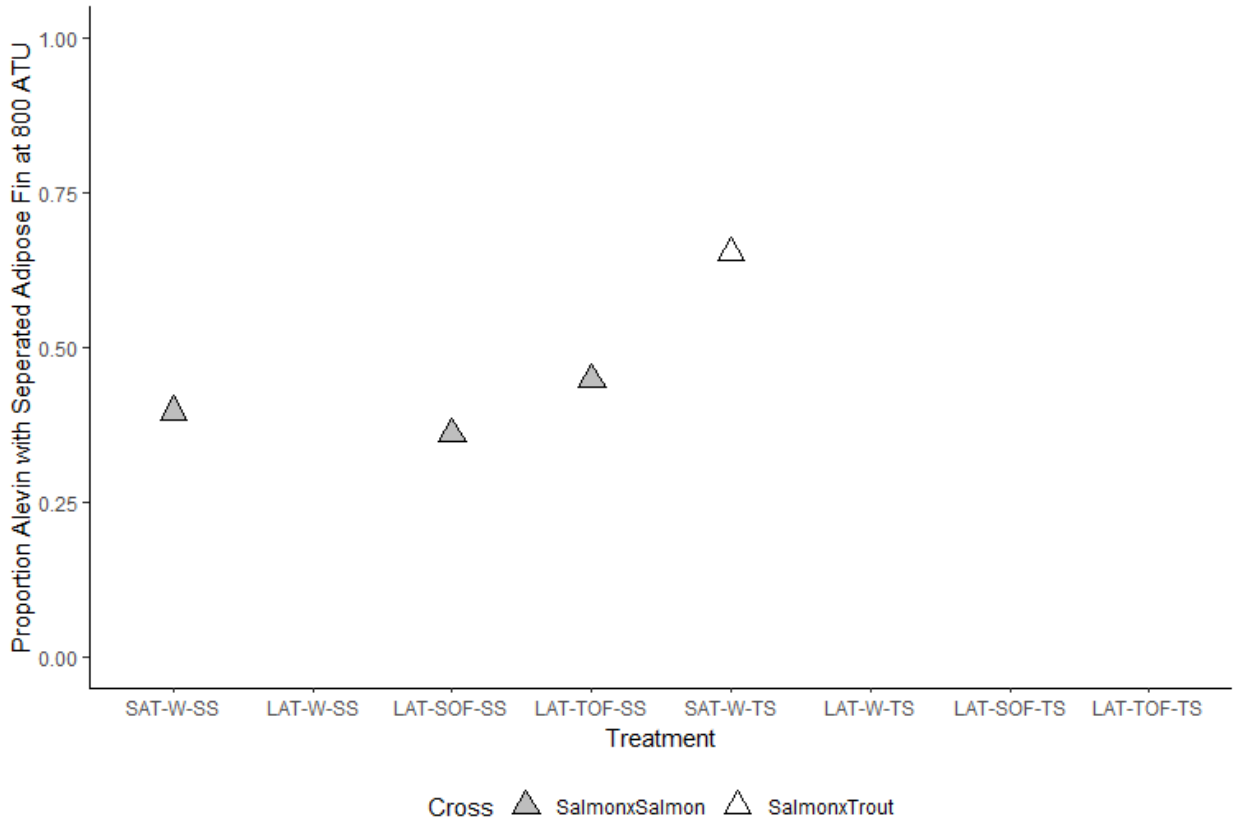
Appendix Figure 3-G2: Proportion of block 2 fish with separated adipose fins at 800 ATU. Triangles represent the means for each treatment. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=80, LAT-W-SS n=128, LAT-SOF-SS n=113, LAT-TOF-SS n=166, SAT-W-TS n=0, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=79. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



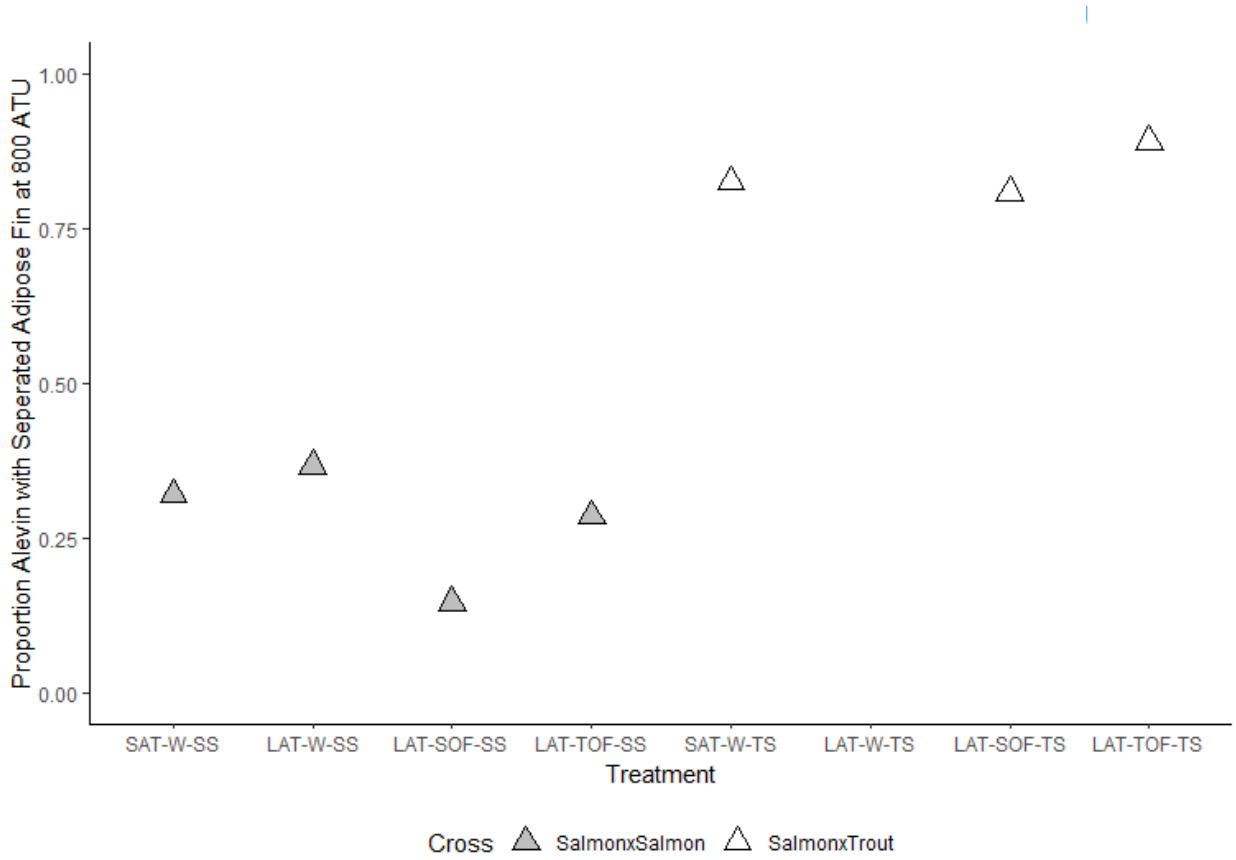
Appendix Figure 3-G3: Proportion of block 3 fish with separated adipose fins at 800 ATU. Triangles represent the means for each treatment. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=49, LAT-W-SS n=0, LAT-SOF-SS n=47, LAT-TOF-SS n=0, SAT-W-TS n=47, LAT-W-TS n=0 LAT-SOF-TS n=47, LAT-TOF-TS n=49. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



Appendix Figure 3-G4: Proportion of block 4 fish with separated adipose fins at 800 ATU. Triangles represent the means for each treatment. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=48, LAT-W-SS n=48, LAT-SOF-SS n=48, LAT-TOF-SS n=48, SAT-W-TS n=37, LAT-W-TS n=0 LAT-SOF-TS n=41, LAT-TOF-TS n=0. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

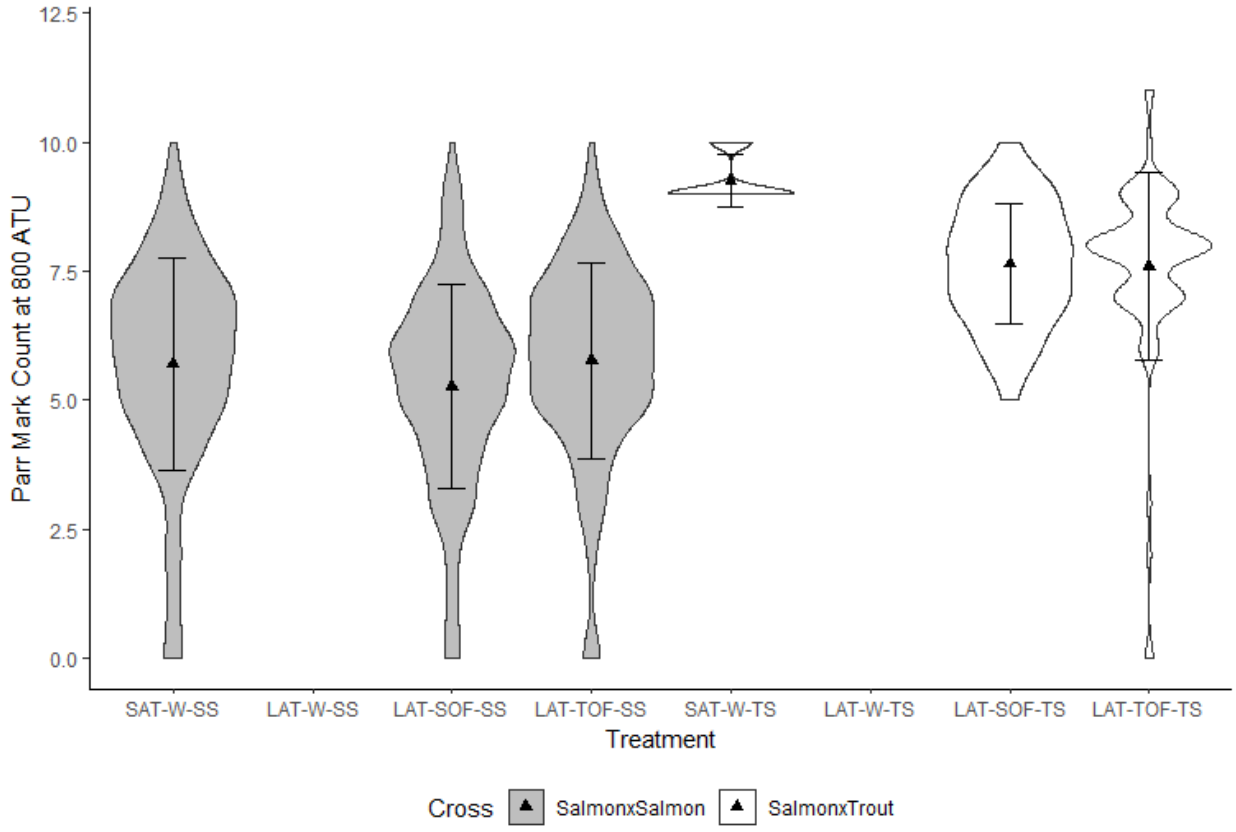


Appendix Figure 3-G5: Proportion of block 5 fish with separated adipose fins at 800 ATU. Triangles represent the means for each treatment. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=48, LAT-W-SS n=0, LAT-SOF-SS n=40, LAT-TOF-SS n=49, SAT-W-TS n=49, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=0. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

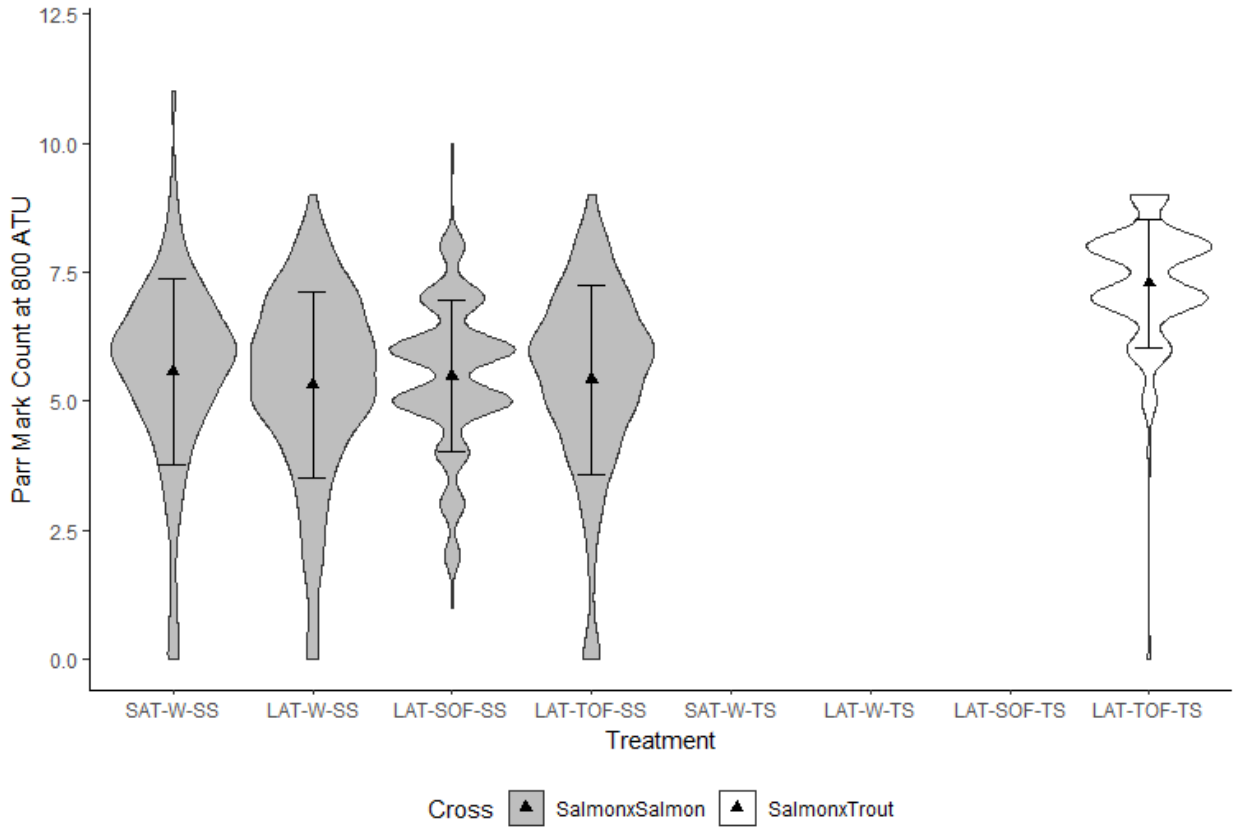


Appendix Figure 3-G6: Proportion of block 6 fish with separated adipose fins at 800 ATU. Triangles represent the means for each treatment. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50 LAT-W-SS n=49, LAT-SOF-SS n=48, LAT-TOF-SS n=49, SAT-W-TS n=50, LAT-W-TS n=0 LAT-SOF-TS n=47, LAT-TOF-TS n=46. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

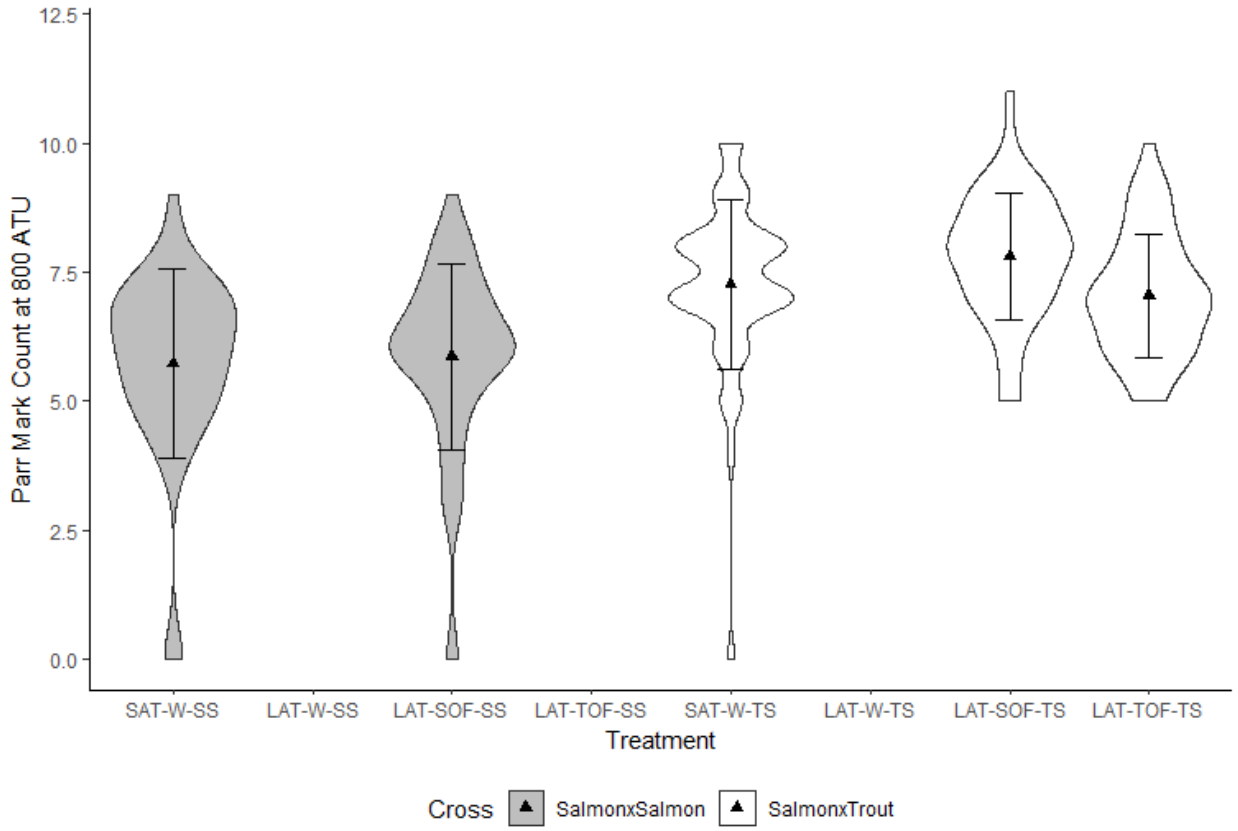
Appendix 3-H: Parr mark count by block



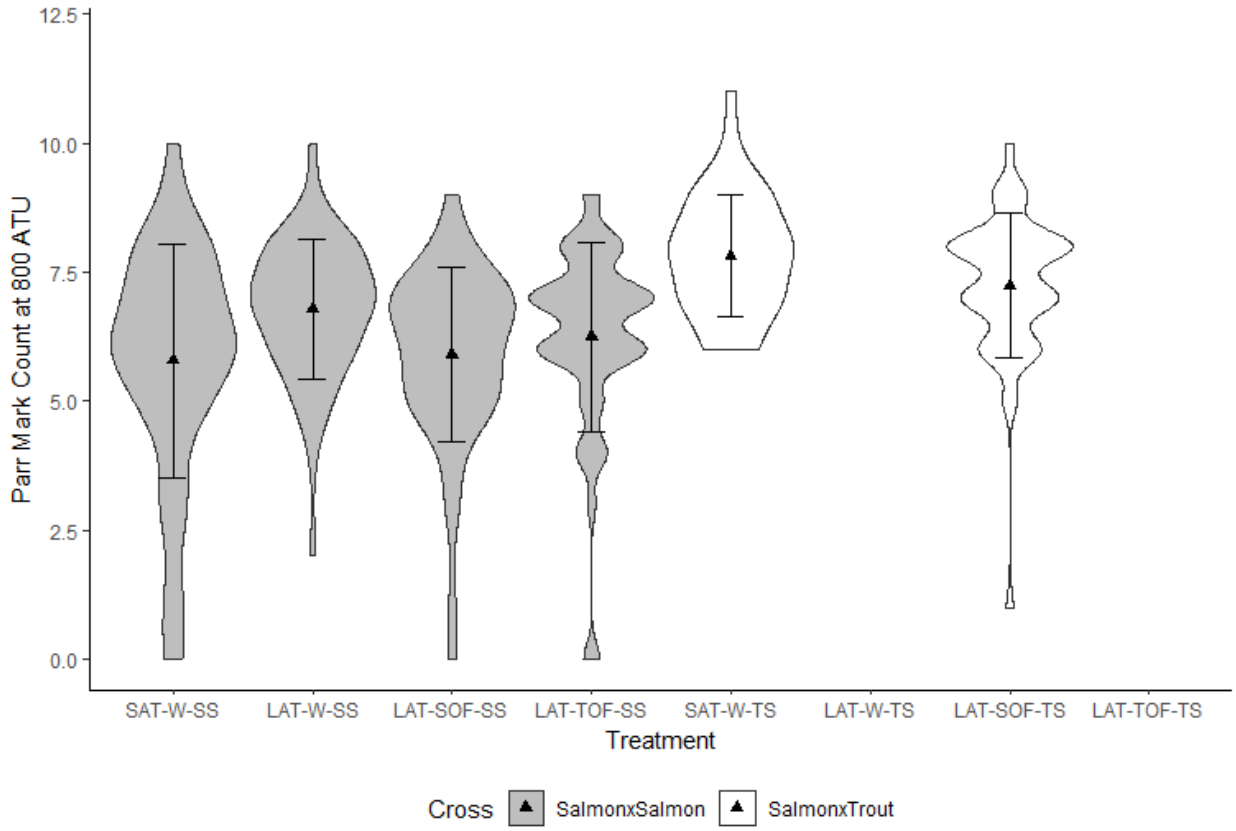
Appendix Figure 3-H1: Parr mark counts from block 1 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=104, LAT-W-SS n=0, LAT-SOF-SS n=174, LAT-TOF-SS n=136, SAT-W-TS n=4, LAT-W-TS n=0 LAT-SOF-TS n=35, LAT-TOF-TS n=77. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



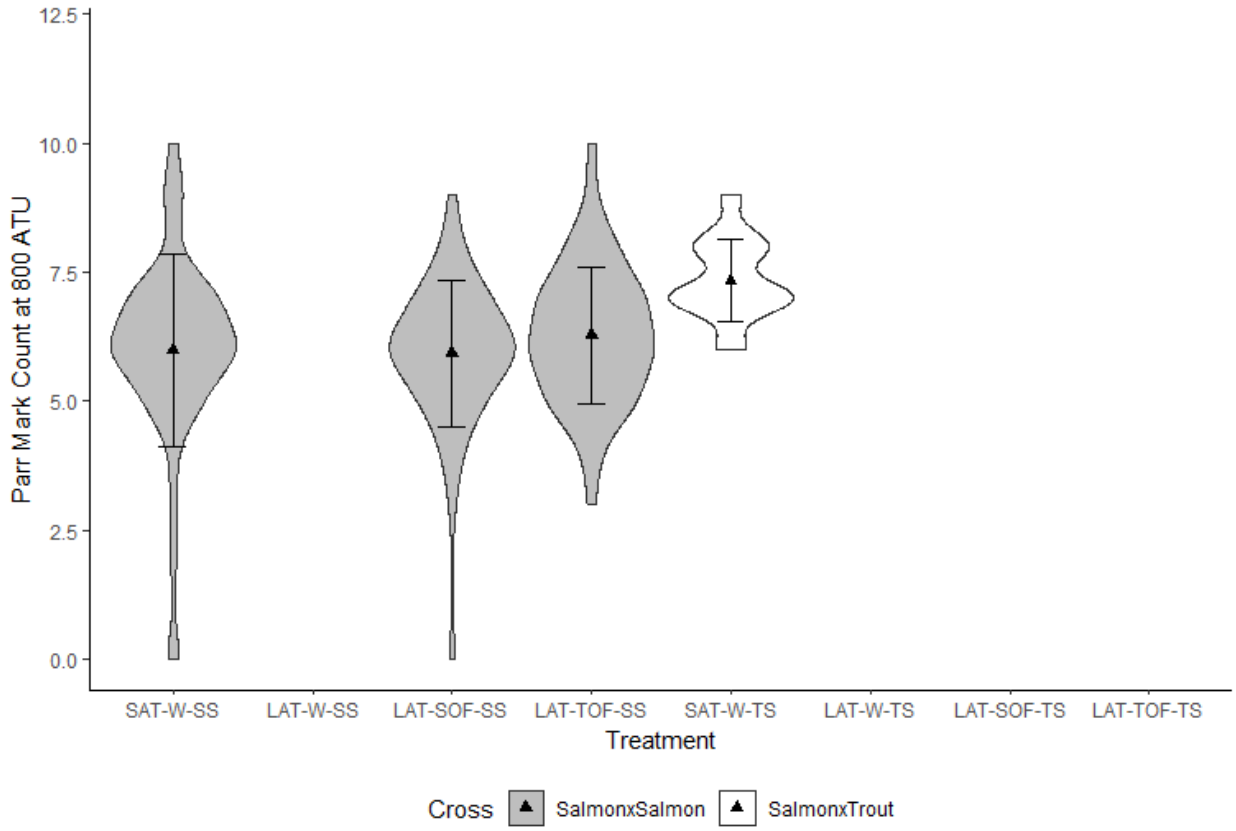
Appendix Figure 3-H2: Parr mark counts from block 2 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=117, LAT-W-SS n=144, LAT-SOF-SS n=135, LAT-TOF-SS n=179, SAT-W-TS n=0, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=116. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



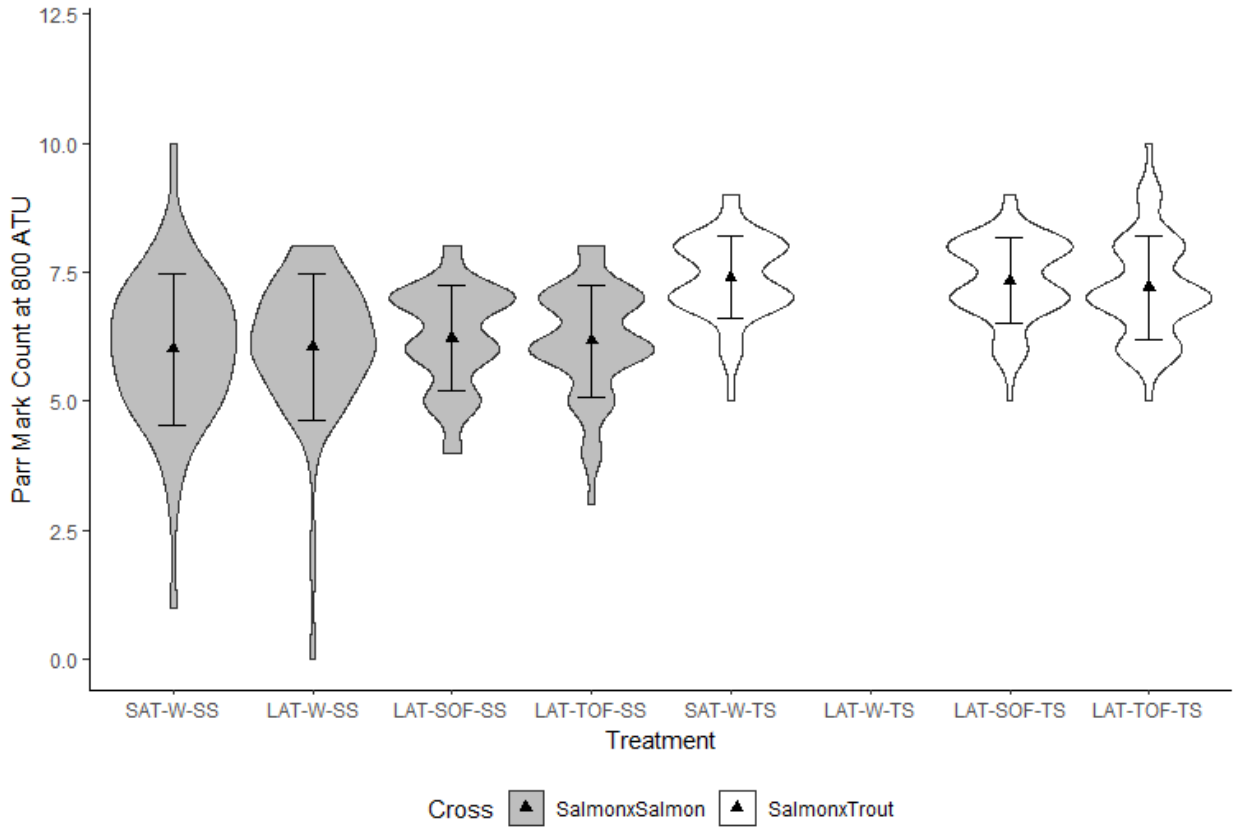
Appendix Figure 3-H3: Parr mark counts from block 3 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=0, LAT-SOF-SS n=50, LAT-TOF-SS n=0, SAT-W-TS n=50, LAT-W-TS n=0 LAT-SOF-TS n=50, LAT-TOF-TS n=50. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



Appendix Figure 3-H4: Parr mark counts from block 4 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=50, LAT-SOF-SS n=50, LAT-TOF-SS n=50, SAT-W-TS n=50, LAT-W-TS n=0 LAT-SOF-TS n=50, LAT-TOF-TS n=0. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

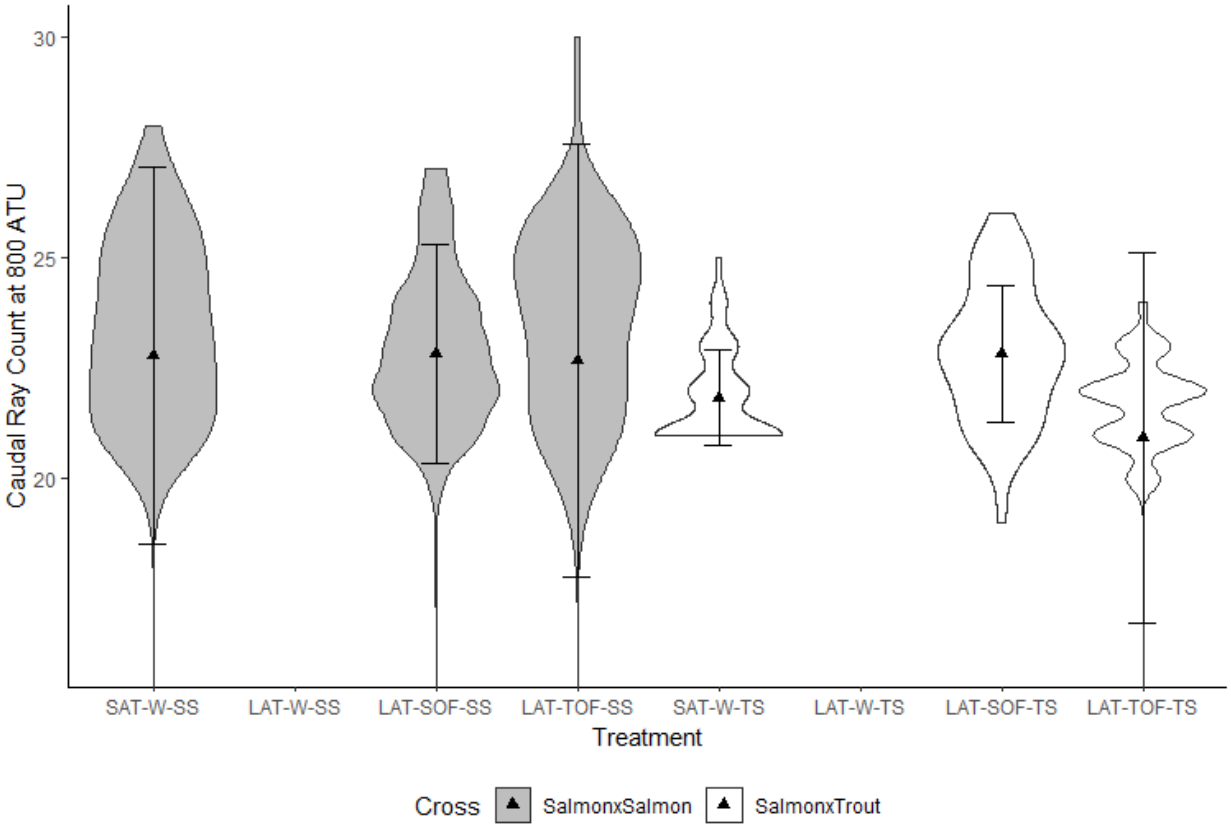


Appendix Figure 3-H5: Parr mark counts from block 5 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=0, LAT-SOF-SS n=50, LAT-TOF-SS n=50, SAT-W-TS n=50, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=0. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

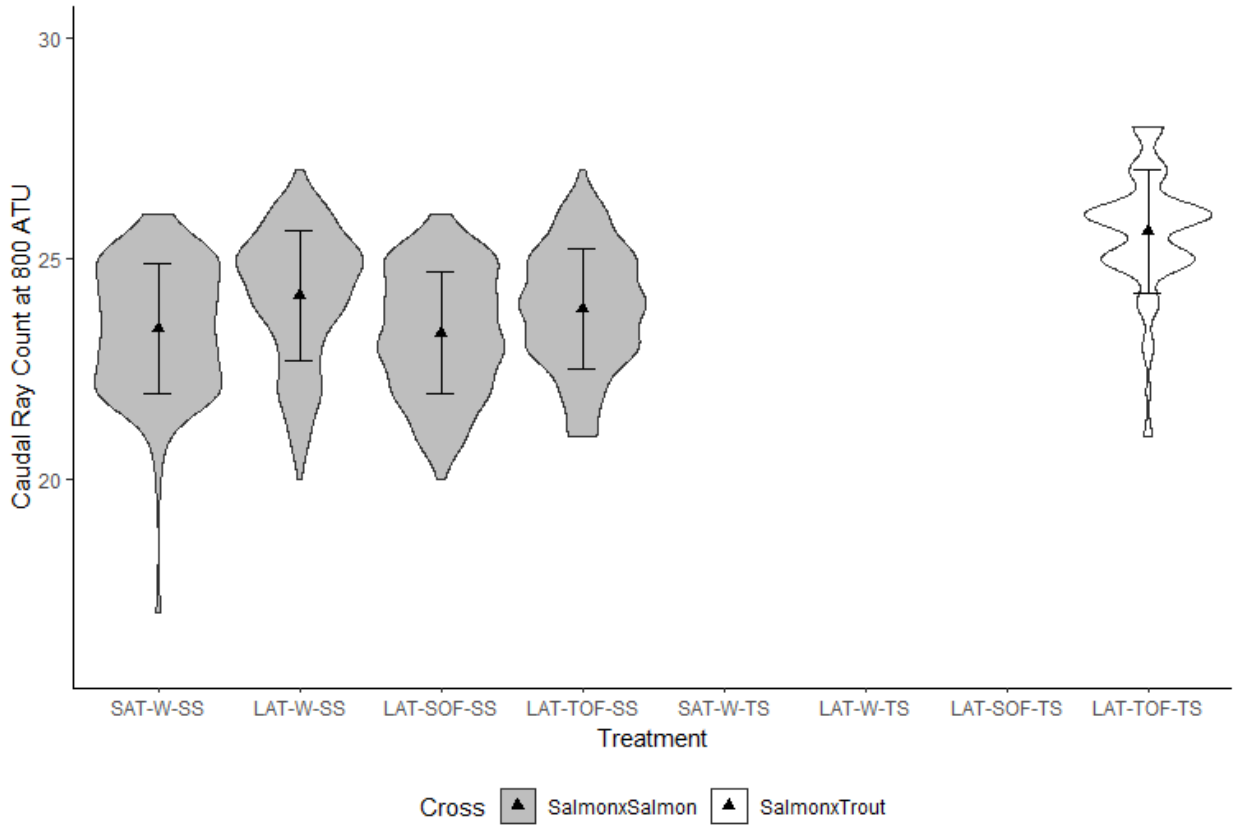


Appendix Figure 3-H6: Parr mark counts from block 6 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=50, LAT-SOF-SS n=50, LAT-TOF-SS n=50, SAT-W-TS n=50, LAT-W-TS n=0 LAT-SOF-TS n=50, LAT-TOF-TS n=50. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

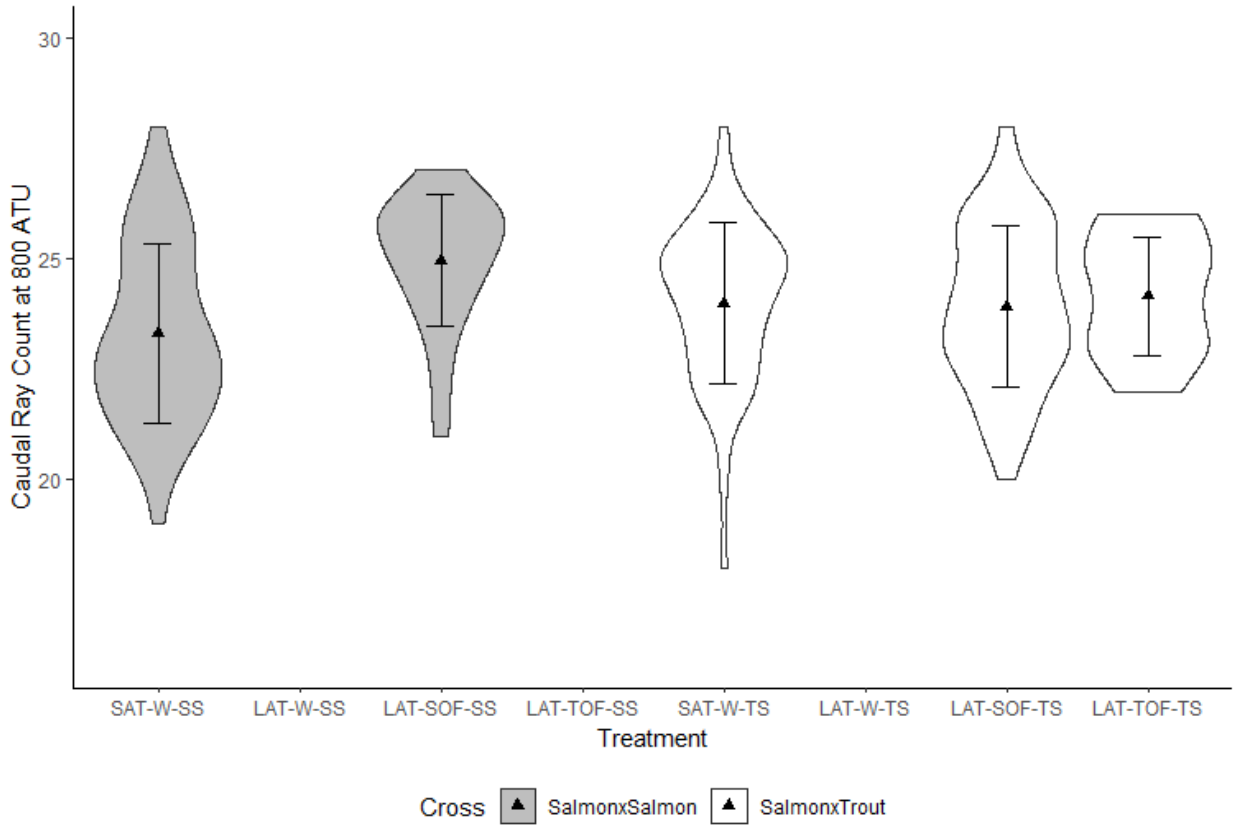
Appendix 3-I: Caudal Ray count at 800 ATU by block



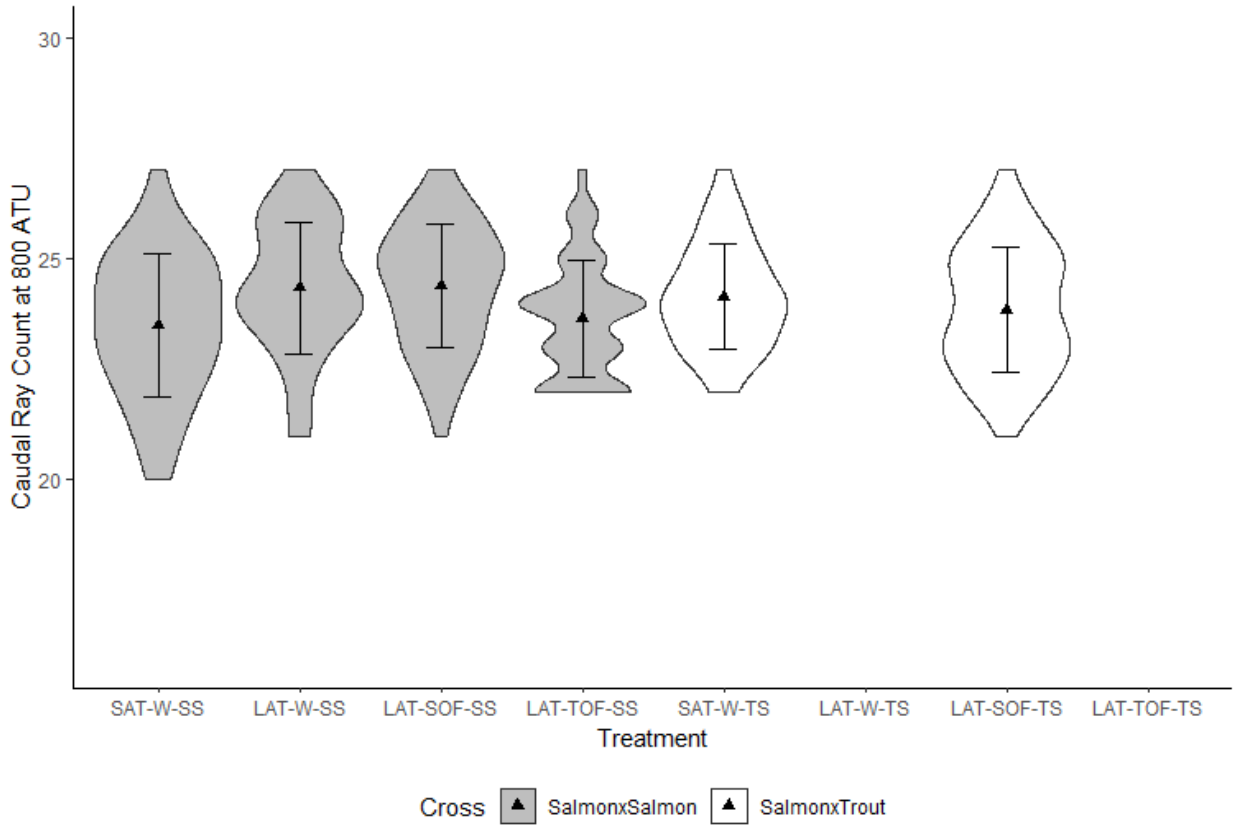
Appendix Figure 3-I1: Caudal ray counts from block 1 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=146, LAT-W-SS n=0, LAT-SOF-SS n=174, LAT-TOF-SS n=131, SAT-W-TS n=45, LAT-W-TS n=0 LAT-SOF-TS n=36, LAT-TOF-TS n=107. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



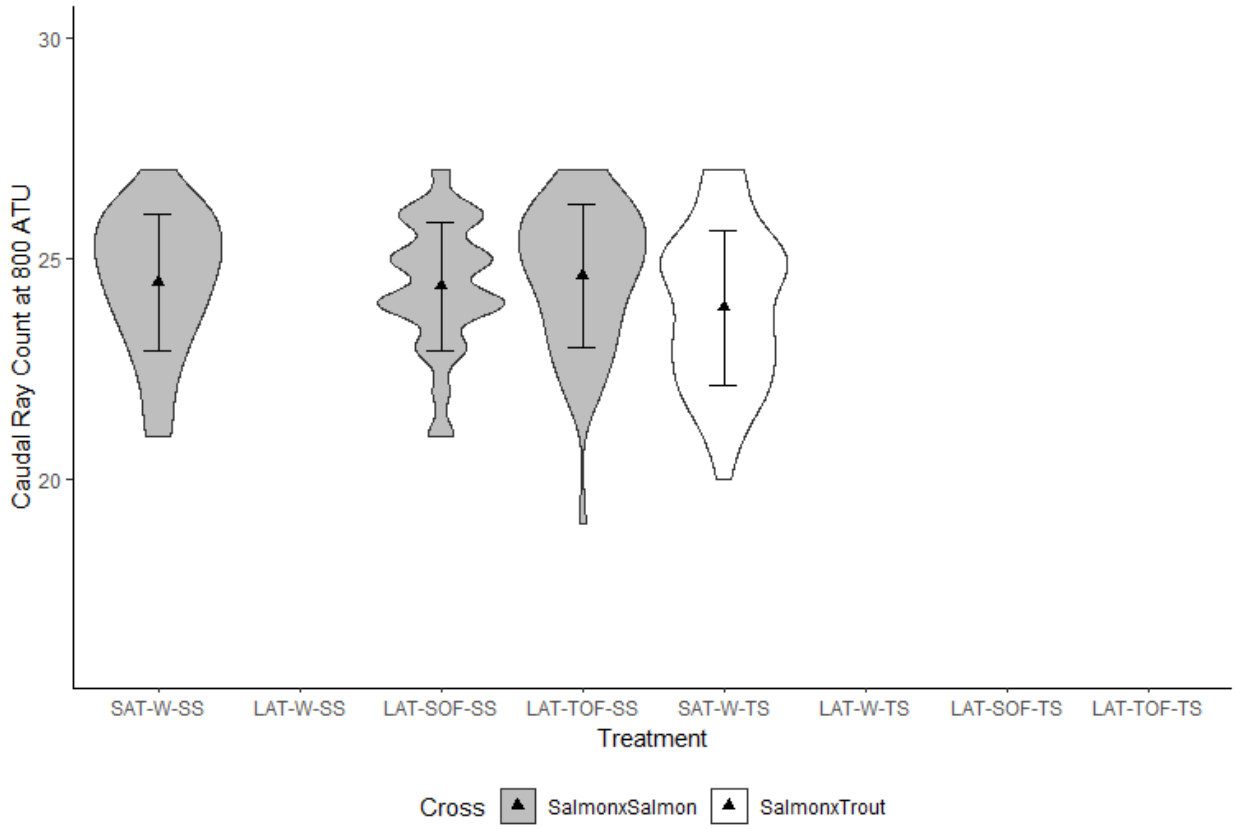
Appendix Figure 3-I2: Caudal ray counts from block 2 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=91, LAT-W-SS n=140, LAT-SOF-SS n=134, LAT-TOF-SS n=174, SAT-W-TS n=0, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=83. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



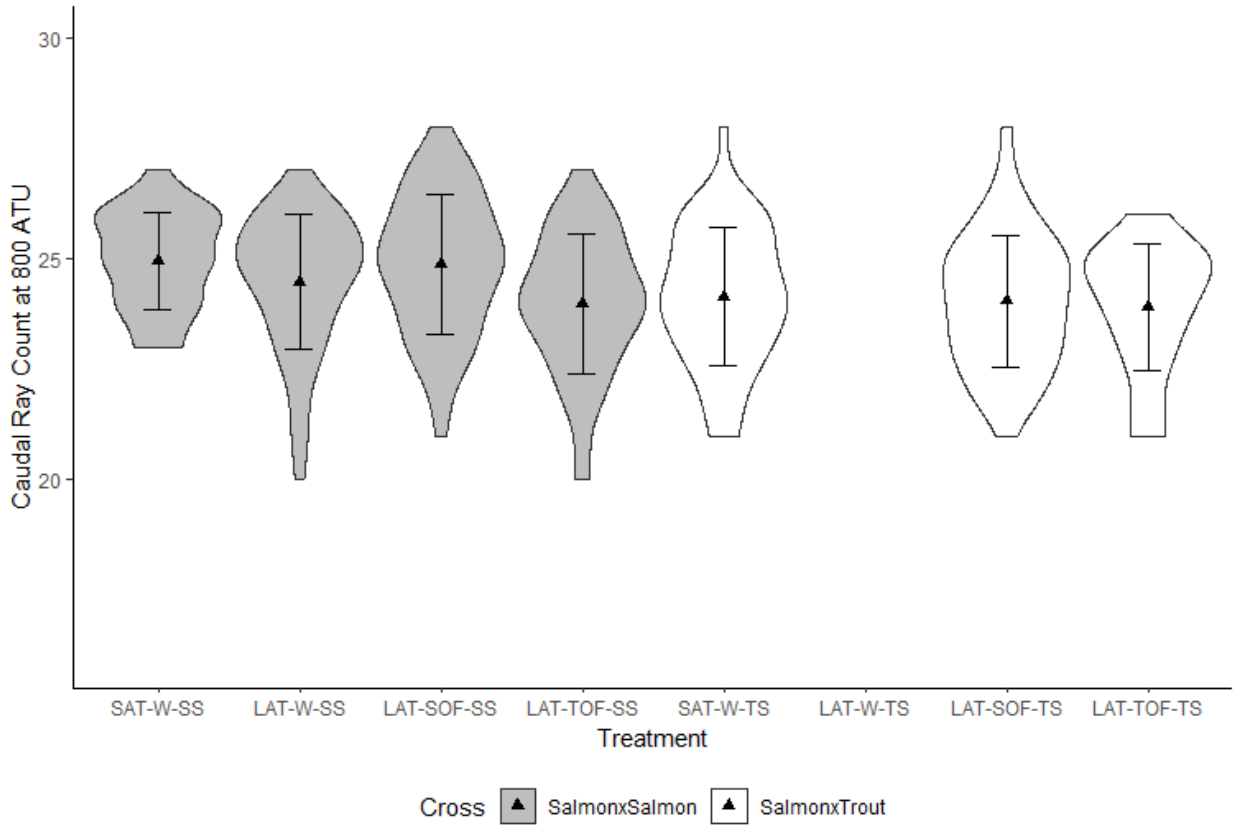
Appendix Figure 3-I3: Caudal ray counts from block 3 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=49, LAT-W-SS n=0, LAT-SOF-SS n=47, LAT-TOF-SS n=0, SAT-W-TS n=49, LAT-W-TS n=0 LAT-SOF-TS n=50, LAT-TOF-TS n=49. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



Appendix Figure 3-I4: Caudal ray counts from block 4 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=49, LAT-W-SS n=49, LAT-SOF-SS n=50, LAT-TOF-SS n=49, SAT-W-TS n=35, LAT-W-TS n=0 LAT-SOF-TS n=48, LAT-TOF-TS n=0. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



Appendix Figure 3-I5: Caudal ray counts from block 5 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=0, LAT-SOF-SS n=50, LAT-TOF-SS n=50, SAT-W-TS n=49, LAT-W-TS n=0 LAT-SOF-TS n=0, LAT-TOF-TS n=0. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.



Appendix Figure 3-I6: Caudal ray counts from block 6 fish at 800 ATU. Triangles represent the means for each treatment, and bars represent standard deviations. The width of the violin shows the frequency of the values within the data. Sample sizes for each treatment were as follows; SAT-W-SS n=50, LAT-W-SS n=48, LAT-SOF-SS n=48, LAT-TOF-SS n=49, SAT-W-TS n=49, LAT-W-TS n=0 LAT-SOF-TS n=47, LAT-TOF-TS n=47. Legend: SAT= Short Activation (0 seconds), LAT=Long Activation (20 seconds), W=Water, SOF=Salmon ovarian fluid, TOF=Trout ovarian fluid.

Chapter 4. Discussion

Aims and interpretation

I sought to examine the potential linkage between cryptic female choice and gamete-mediated paternal effects in the context of interspecific hybridization. In these study species, ovarian fluid mediated changes to sperm swimming behavior have been previously reported to act as the operating force of cryptic female choice. Both conspecific sperm preference (Yeates et al., 2013) and gamete-mediated paternal effects (Immler et al., 2014) have been documented in salmonids, but the intersection of these concepts has not yet been studied. Following previous work, I expected to find significant effects of conspecific sperm preference on both sperm behaviour and gamete-mediated paternal effects on offspring, especially with the cost associated with wasted gametes as a function of hybridization. Instead, I got some unexpected results that go against what has been previously published on certain populations of these study species. In this chapter, I discuss the unexpected results in comparison to previous studies and present the factors that played into them.

When premating isolation, either from physical isolation (temporal and/or spatial) or behavioural (mate choice) breaks down, post-ejaculatory pre-zygotic sexual section such as cryptic female choice is the last mechanism that can stop fertilization from a heterospecific male (Devigili et al., 2018; Howard et al., 2009). There are strong examples of cryptic female choice across taxa, including salmonids, that can explicitly prevent fertilization from the non-preferred male (Birkhead & Pizzari, 2002; Manier et al., 2013; Tyler et al., 2013; Yeates et al.,

2013). My research described in the second chapter of this thesis used two well-studied species, Atlantic salmon and brown trout, whose European populations exhibit strong cryptic female choice (Yeates et al., 2013). I further improved upon this design by adding a third species, brook char, which has been documented to hybridize with both salmon and trout to varying degrees of success (Buss & Wright, 1958; Chevassus, 1979). In doing so, I expected to discern a pattern of ovarian fluid mediated upregulation, where all sperm was upregulated by ovarian fluid, but conspecific sperm was upregulated more than heterospecific sperm. Among heterospecific sperm, I hypothesized there would be a pattern of upregulation depending on the genetic distance between the species or the likelihood of interaction in natural spawning conditions.

I confirmed that ovarian fluid from any of our study species upregulated the proportion of sperm motile and the sperm swimming velocity across all species compared to water by 54 and 30 percent, respectively. This confirms that ovarian fluid does upregulate sperm behavior regardless of sperm species, likely due to the chemical components within ovarian fluid. These chemicals have been documented to maintain sperm motility, lengthen swimming time (Elofsson et al., 2006), and increase sperm velocity (Butts et al., 2017) in both our study populations (Purchase & Rooke, 2020) and other study taxa and systems. In a surprising contrast to existing studies in other populations of our study species (Yeates et al., 2013), we were unable to find a significant upregulation of conspecific sperm versus heterospecific sperm by ovarian fluid. When broken down to the species level, there was only a meaningful upregulation (34 percent) in salmon ovarian fluid between two different species of heterospecific sperm, trout and char. The lack of relevant biological effects linked to experience

in ovarian fluid to conspecific vs. heterospecific sperm may explain some of my results in Chapter 3.

Following research done on Atlantic salmon that found that the time sperm swam prior to contact with eggs affected offspring development (Immler et al., 2014), I expected to (1) repeat these findings and (2) determine that the medium that sperm swam in before fertilization (conspecific and heterospecific ovarian fluid vs. water) would also affect offspring development. I expanded upon the Immler et al. (2014) study and created hybrids to compare the effects of sperm experience and hybridization. I expected and found that effects from hybridization (average of 10.7% difference) were much greater than those from gamete-mediated parental effects (average of 1.4% difference). Hybrids hatched faster and were larger no matter what sperm were exposed to before fertilization. When compared to effects linked from hybridization, gamete-mediated paternal effects were not biologically relevant. It was unexpected that the act of sperm swimming and the medium they swam in did not appear to affect development because of the extreme difference in sperm swimming behaviour compared to ovarian fluid vs. water. While the result in Chapter 2 on conspecific sperm preference could explain why paternal effects were so weak in the comparison between conspecific and heterospecific ovarian fluid, it does not explain why did not see drastic paternal effects resulting from the strong modification of sperm in ovarian fluid vs. water. This implies that in this system, females are free to modify paternity through cryptic female choice with no consequences to the offspring's development. These differences from previously examined populations can be potentially explained by looking at the differences between previously studied systems and our study system.

Differences between study systems and past studies

Prior work has uncovered strong conspecific sperm preference in naturally sympatric Atlantic salmon and brown trout in Europe (Yeates et al., 2013). Given the impacts of hybridization, it was expected that this mechanism would also be in place in situations of invasion. While I replicated the upregulation of sperm swimming behaviour linked to ovarian fluid compared to water described in previous work (Butts et al., 2017; Elofsson et al., 2006), I could not find support for conspecific sperm preference. This is very surprising due to the existing patterns in Europe (Yeates et al., 2013) as well as the high cost evolutionarily of having hybrid offspring (Pampoulie et al., 2021; Trivers, 1972). The fact that brown trout were introduced and are invasive to North America (MacCrimmon and Marshall, 1968) may be responsible for this unexpected result since these mechanisms would not have had as much time to evolve in native Newfoundland Atlantic salmon. Newfoundland Atlantic salmon have been reproductively isolated from European Atlantic salmon for 600,000 years (Lehnert et al., 2020). Because of this, interactions between Atlantic salmon and brown trout on both continents could be very different. This isolation could diminish the importance of strong conspecific preference and cause those traits to not be selected for in the population. Despite this, it is important to note that the same dynamic did not appear between native brook char and Atlantic salmon, who were not spatially or temporally isolated on the species level but on the population level (as Star Lake brook char have had no exposure to Atlantic salmon even though they occur in the same watershed).

There was no notable effect of sperm swimming medium or sperm swimming on salmon or hybrid salmon-trout offspring development, even though ovarian fluid drastically changes sperm behavior when compared to water. While I found a sizable difference between hybrids and non-hybrids at hatch and at 800 accumulated temperature units, I did not find a consistent biologically significant difference between groups fertilized with sperm exposed to different environments and times. It is surprising that we were unable to duplicate prior work that found that the mere act of sperm swimming was linked to changes in offspring development in prior work on European Atlantic salmon (Immler et al., 2014). The fact that I was unable to replicate these results with either of these study populations raises questions about the effects of sperm experience on offspring. It is uncertain what could have brought about this inability to replicate previous results in salmon, particularly since our fertilization protocols were designed following the Immler et al. (2014) work. Potentially, differences between European and North American salmon and brown trout could have caused these unexpected results.

When European brown trout and Atlantic salmon hybridize in the wild, the offspring that are found originate from matings between salmon mothers and trout fathers (Garcia-Vasquez et al., 2004). In Newfoundland, the opposite is true, and surviving juveniles are produced by trout fathers and salmon mothers (McGowan & Davidson, 1992; Poulos, 2019). Laboratory studies show, however, that both crosses create viable offspring with relatively high survival (Chevassus, 1979, Poulos, 2019). European and North American Atlantic salmon have different numbers of chromosomes, which can change the viability of hybrid offspring (Hartley, 1987). While I examined both directions of this cross in Chapter 2, I did not examine both directions of the crosses in Chapter 3. However, this should not have caused our lack of effect

across our treatments. I do not believe that there were any underlying issues in the study design that would explain the differences between our results and those in pre-existing literature.

My studies were well designed to examine the magnitude and effects of conspecific sperm preference and paternal effects on offspring development. In contrast to the Yeates et al. (2013) work with conspecific sperm preference, we sampled more individuals for sperm swimming comparisons and did more crosses as a result of adding a third species to the study. This should not have been responsible for our inability to replicate their results. I did have issues in the paternal effects work because of lower-than-expected survival in our LAT-W treatments, which then limited the comparisons I was able to make across treatments between salmon-trout hybrids and salmon. However, given the biologically irrelevant results I got in the salmon treatments and the trends between hybrids and salmon in the other three treatments (SAT-W, LAT-SOF, and LAT-TOF), I could expect a similar result in the LAT-W treatment for hybrids and salmon. In contrast to the Immler et al. (2014) work with paternal effects, we opted to add more treatments and kept our fish for more ATU's to give the paternal genome more time to express. Incubation was also done at a constant temperature vs. a natural river temperature to slow development and make changes to development related to treatment more evident. These changes to the experimental design should have made any patterns easier to identify, and therefore would not be responsible for our lack of apparent gamete-mediated paternal effects. Given the consequences of reproducing with a heterospecific male, it is surprising that both conspecific sperm preference and gamete mediated paternal effects as a result of strong conspecific sperm preference were absent.

Consequences of reproductive interaction

While Atlantic salmon and brown trout (Yeates et al., 2013) and brook char and brown trout (Sorensen et al., 1995) have a high likelihood of using the same spawning habitat at overlapping times; Atlantic salmon and brook char do not. Brook char spawn earlier than Atlantic salmon and brown trout (McGowan & Davidson, 1992; O'Connell, 1982), which implies that any reproductive interactions between these species would be between late char and early salmon (typically sneaker males in the case of salmon). In situations where char do overlap, there have been documented cases of other species of salmonids (in this case, brown trout) upending or spawning over brook char redds (Sorensen et al., 1995). This has been linked to trout displacement of char in North America. Spawning overlap also leads to potential hybridization, which can have drastic impacts on reproducing individuals and populations.

Eggs fertilized by a heterospecific male represent an evolutionary waste in the sense that those eggs will not be the same species as their mother. Depending on the direction of fertilization (sneaker heterospecific males fertilizing heterospecific eggs, or dominant heterospecific males outcompeting smaller conspecific males) this can either slow or increase the rate of hybridization in these systems (Garner & Neff, 2013; Tynkkynen et al., 2009). In systems with invading salmonids such as brown trout in North America, this could also play into driving further invasion. If brown trout males can outcompete native species for fertilizations on the spawning grounds while outcompeting native males in attempting to mate with their conspecific females, the invasive trout could potentially displace or extirpate these native populations through the forcing of evolutionary dead ends and increase the impacts of

invasion. Given these high stakes associated with hybridization, it is surprising that conspecific sperm preference is absent in this study system. Given that I did not replicate prior results with both experiments, more work of this nature should be undertaken to fully parse out the effects of sperm swimming experience on paternity and paternal effects in the context of hybridization.

Next steps

In the big picture, the impact of these revelations is that salmonids are more complicated regarding post-mating pre-fertilization sexual selection than previously thought. Strong cryptic female choice and clear parental effects have been well documented in the salmonids both within and across species (Immler et al., 2014; Rosengrave et al., 2016; Yeates et al., 2013). Clearly, expanding on this previous work has created discrepancies resulting from examining different populations and examining more species. With the spread of brown trout across the world (Westley & Fleming, 2011), the ability to examine these mechanisms across a variety of sympatric and allopatric distributions is likely the most valuable next step to take. This would determine if there was an adaptive component to cryptic female choice, where females in sympatry would face selection for strong cryptic female choice. If that were the case, perhaps gamete-mediated paternal effects would also be stronger and more biologically relevant to reflect the more robust alteration to sperm swimming behaviour. In line with sympatry and allopatry, consideration should be taken to examine systems with novel invasions of brown trout compared to those that have had invasive brown trout for longer periods of time. Comparing those types of systems would then provide evidence for or against this

mechanism evolving over time. Examining pre-mating and pre-fertilization sexual selection, both from males and females, could provide insight into the extent and need for cryptic female choice in these systems.

Weak post-mating pre-fertilization isolation could point to strong pre-mating isolation in some salmonid systems; where females are able to avoid releasing eggs when courted by a heterospecific male. For example, brown trout females in Minnesota were documented to not release gametes when courted by a brook char male (Sorensen et al., 1995). If present, dominant conspecific males are also able to limit sneaker males' access to females before they can steal fertilizations (Morbey, 2002; Sorensen et al., 1995). When sneakers are not part of the equation this could effectively eliminate interspecific hybridization. While temporal or spatial isolation is not likely to occur on the spawning grounds, behavioural selection, particularly by females for members of her own species, could be strong enough to compensate for weak cryptic female choice. Other hybridizing salmonids should also be considered in examining hybridization in this taxon.

Species of *Oncorhynchus* and *Salvelinus* also have been documented to hybridize both in the aquaculture setting and the wild (Chevassus, 1979; Ito et al., 2006). Hybrids are common within both genera, with most crosses producing offspring and some (particularly within *Oncorhynchus* and *Salvelinus*) fertile offspring (Chevassus, 1979). Hybrids between rainbow trout (*Oncorhynchus mykiss*) and cutthroat trout (*Oncorhynchus clarkii*), known as cutbows, are of particular interest because offspring are fertile (Parker et al., 2011). Intergeneric hybrids are much less common; notable examples of crosses with high offspring survival include those

between Arctic char and brown trout, Arctic char and Atlantic salmon, and biwa trout (*Oncorhynchus rhodurus*) and Dolly Varden (*Salvelinus malma*). However, in situations of intergeneric hybridization, high survival is not the norm. (Chevassus, 1979). Studies of this type should also be done on other members of the salmonids due to the commonality of hybridization.

The genus Oncorhynchus would be a good fit for examining conspecific sperm preference and paternal effects linked to hybridization in assemblages that have been exposed to one another for long periods of time because hybrids are common (Chevassus, 1979; Ito et al., 2006) . In these assemblages, one would expect to see strong conspecific sperm preference due to long exposure to one another on the spawning grounds. While brown trout have a reputation for being a strong invader (Macrimmon and Marshall, 1968; Hustins, 2007) other species such as rainbow trout (also have invaded watersheds around the world (Crawford and Muir, 2008). This dynamic would also be worth investigating as heterogeneric hybrids are also common between invasive rainbow trout and native species (Parker et al., 2011). Given that invasion exists on a gradient, sampling a variety of sites and populations within these species should produce a meaningful comparison across differing degrees of sympatry and allopatry. Salmonid behavior also creates opportunities to explore this interaction before mating takes place.

Salmonids have been shown to exhibit strong mate choice in the face of hybridization. Further studies of mate selection behavior in spawning habitat, coupled with supplementary work on pre-mating isolation, would help to further flesh out this female perspective of

hybridization. Examining males in this context, both in sperm competition and male-male competition for mates, would also help uncover the actual conflict of hybridization that the female faces. This, coupled with widespread alternative reproductive tactics in salmonids (Blanchfield et al., 2003; Garcia-Vazquez et al., 2002; Kolm et al., 2009), shows the need for a more inclusive picture of this mating system. Further examination of the differences between populations of these fish would also help to advance the knowledge of paternal effects in the context of hybridization.

It is the author's opinion that continuing work on pre- and post-mating barriers to hybridization in salmonids, as well as the resulting gamete-mediated paternal effects in this system, should be continued. The complexities between the interactions between spawning individuals of different species as well as the accessibility of assemblages of salmonid species make them a unique taxon that is well suited to examining these questions.

Literature cited

Birkhead, T. R., & Pizzari, T. (2002). Postcopulatory sexual selection. *Nature Reviews. Genetics*, *3*(4), 262–273. https://doi.org/10.1038/nrg774

Blanchfield, P. J., Ridgway, M. S., & Wilson, C. C. (2003). Breeding success of male brook trout (*Salvelinus fontinalis*) in the wild. *Molecular Ecology*, *12*(9), 2417–2428. https://doi.org/10.1046/j.1365-294X.2003.01917.x

Buss, K., & Wright, J. E. (1958). Appearance and fertility of trout hybrids. *Transactions of the American Fisheries Society*, *87*(1), 172–181. https://doi.org/10.1577/1548-8659(1957)87[172:AAFOTH]2.0.CO;2

Butts, I. A. E., Prokopchuk, G., Kašpar, V., Cosson, J., & Pitcher, T. E. (2017). Ovarian fluid impacts flagellar beating and biomechanical metrics of sperm between alternative reproductive tactics. *Journal of Experimental Biology*, *220*(12), 2210–2217. https://doi.org/10.1242/jeb.154195

Chevassus, B. (1979). Hybridization in salmonids: Results and perspectives. *Aquaculture*, *17*(2), 113–128. https://doi.org/10.1016/0044-8486(79)90047-4

Crawford, S. S., & Muir, A. M. (2008). Global introductions of salmon and trout in the genus Oncorhynchus: 1870–2007. *Reviews in Fish Biology and Fisheries*, *18*(3), 313–344. https://doi.org/10.1007/s11160-007-9079-1

Devigili, A., Fitzpatrick, J. L., Gasparini, C., Ramnarine, I. W., Pilastro, A., & Evans, J. P. (2018). Possible glimpses into early speciation: The effect of ovarian fluid on sperm velocity accords with post-copulatory isolation between two guppy populations. *Journal of Evolutionary Biology*, *31*(1), 66–74. https://doi.org/10.1111/jeb.13194

Elofsson, H., Look, K. J. W. V., Sundell, K., Sundh, H., & Borg, B. (2006). Stickleback sperm saved by salt in ovarian fluid. *Journal of Experimental Biology*, *209*(21), 4230–4237. https://doi.org/10.1242/jeb.02481

Garcia-Vazquez, E., Moran, P., Perez, J., Martinez, J. L., Izquierdo, J. I., de Gaudemar, B., & Beall, E. (2002). Interspecific barriers between salmonids when hybridisation is due to sneak mating. *Heredity*, *89*(4), 288–292. https://doi.org/10.1038/sj.hdy.6800134

Garcia-Vazquez, Eva, Perez, J., Ayllon, F., Martinez, J. L., Glise, S., & Beall, E. (2004). Asymmetry of post-F1 interspecific reproductive barriers among brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*). *Aquaculture*, *234*(1), 77–84. https://doi.org/10.1016/j.aquaculture.2004.01.017

Garner, S. R., & Neff, B. D. (2013). Alternative male reproductive tactics drive asymmetrical hybridization between sunfishes (*Lepomis spp.*). *Biology Letters*, *9*(6), 20130658. https://doi.org/10.1098/rsbl.2013.0658

Hartley, S. E. (1987). The chromosomes of salmonid fishes. *Biological Reviews*, *62*(3), 197–214. https://doi.org/10.1111/j.1469-185X.1987.tb00663.x

Howard, D., Palumbi, S., Birge, L., & Manier, M. (2009). Sperm and speciation. In *Sperm Biology* (pp. 367–403). https://doi.org/10.1016/B978-0-12-372568-4.00009-4

Immler, S., Hotzy, C., Alavioon, G., Petersson, E., & Arnqvist, G. (2014). Sperm variation within a single ejaculate affects offspring development in Atlantic salmon. *Biology Letters*, *10*(2), 20131040. https://doi.org/10.1098/rsbl.2013.1040

Ito, D., Fujiwara, A., & Abe, S. (2006). Hybrid inviability and chromosome abnormality in salmonid fish. *The Journal of Animal Genetics*, *34*(1), 65–70. https://doi.org/10.5924/abgri2000.34.65

Kolm, N., Gonzalez-Voyer, A., Brelin, D., & Winberg, S. (2009). Evidence for small scale variation in the vertebrate brain: Mating strategy and sex affect brain size and structure in wild brown trout (*Salmo trutta*). *Journal of Evolutionary Biology*, *22*(12), 2524–2531. https://doi.org/10.1111/j.1420-9101.2009.01875.x

Lehnert, S. J., Kess, T., Bentzen, P., Clément, M., & Bradbury, I. R. (2020). Divergent and linked selection shape patterns of genomic differentiation between European and North American Atlantic salmon (*Salmo salar*). *Molecular Ecology*, *29*(12), 2160–2175. https://doi.org/10.1111/mec.15480

MacCrimmon, H. R., & Marshall, T. L. (1968). World Distribution of Brown Trout, Salmo trutta. *Journal of the Fisheries Board of Canada*. https://doi.org/10.1139/f68-225

Manier, M. K., Lüpold, S., Belote, J. M., Starmer, W. T., Berben, K. S., Ala-Honkola, O., Collins, W. F., & Pitnick, S. (2013). Postcopulatory sexual selection generates speciation phenotypes in *Drosophila*. *Current Biology: CB*, *23*(19), 1853–1862. https://doi.org/10.1016/j.cub.2013.07.086

McGowan, C., & Davidson, W. S. (1992). Unidirectional natural hybridization between brown trout (*Salmo trutta*) and Atlantic Salmon (*Salmo salar*) in Newfoundland. *Canadian Journal of Fisheries and Aquatic Sciences*, *49*(9), 1953–1958. https://doi.org/10.1139/f92-216

Morbey, Y. E. (2002). The mate-guarding behaviour of male kokanee (*Oncorhynchus nerka*). *Behaviour*, *139*(4), 507–528. www.jstor.org/stable/4535935.

O'Connell, M. F. (Michael F. (1982). The biology of anadromous *Salvelinus fontinalis* (Mitchill, 1815) and *Salmo trutta* Linnaeus, 1758 in river systems flowing into Placentia Bay and St. Mary's Bay, Newfoundland [Doctoral, Memorial University of Newfoundland]. https://research.library.mun.ca/4191/

Ortíz-Barrientos, D., Counterman, B. A., & Noor, M. A. F. (2007). Gene expression divergence and the origin of hybrid dysfunctions. *Genetica*, *129*(1), 71–81. https://doi.org/10.1007/s10709-006-0034-1

Pampoulie, C., Gíslason, D., Ólafsdóttir, G., Chosson, V., Halldórsson, S. D., Mariani, S., Elvarsson, B. Þ., Rasmussen, M. H., Iversen, M. R., Daníelsdóttir, A. K., & Víkingsson, G. A. (2021). Evidence of unidirectional hybridization and second-generation adult hybrid between the two largest animals on Earth, the fin and blue whales. *Evolutionary Applications*, *14*(2), 314–321. https://doi.org/10.1111/eva.13091

Parker, D., Avers, T., & Courtney, M. (2011). Weight, Length, and Growth in Cutbow Trout (Oncorhynchus mykiss x clarkii). *Nature Precedings*. https://doi.org/10.1038/npre.2011.6432.1

Poulos, S. (2019). Embryo development under context of hybridization and a review of regional patterns in hybrid frequency of Atlantic salmon and brown trout [Masters, Memorial University of Newfoundland]. https://research.library.mun.ca/13969/

Purchase, C. F., & Rooke, A. C. (2020). Freezing ovarian fluid does not alter how it affects fish sperm swimming performance: Creating a cryptic female choice 'spice rack' for use in

split-ejaculate experimentation. *Journal of Fish Biology*, *96*(3), 693–699. https://doi.org/10.1111/jfb.14263

Rosengrave, P., Montgomerie, R., & Gemmell, N. (2016). Cryptic female choice enhances fertilization success and embryo survival in chinook salmon. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1827), 20160001. https://doi.org/10.1098/rspb.2016.0001

Schumer, M., Cui, R., Rosenthal, G. G., & Andolfatto, P. (2015). Reproductive isolation of hybrid populations driven by genetic incompatibilities. *PLoS Genetics*, *11*(3), e1005041. https://doi.org/10.1371/journal.pgen.1005041

Sorensen, P. W., Essington, T., Weigel, D. E., & Cardwell, J. R. (1995). Reproductive interactions between sympatric brook and brown trout in a small Minnesota stream. *Canadian Journal of Fisheries and Aquatic Sciences*, *52*(9), 1958–1965. https://doi.org/10.1139/f95-787

Trivers, R. (1972). Parental investment and sexual selection. In *Sexual Selection and the Descent of Man* (p. 378).

Tyler, F., Harrison, X. A., Bretman, A., Veen, T., Rodríguez-Muñoz, R., & Tregenza, T. (2013). Multiple post-mating barriers to hybridization in field crickets. *Molecular Ecology*, *22*(6), 1640–1649. https://doi.org/10.1111/mec.12187

Tynkkynen, K., Raatikainen, K. J., Häkkilä, M., Haukilehto, E., & Kotiaho, J. S. (2009). Alternative reproductive tactics and the propensity of hybridization. *Journal of Evolutionary Biology*, *22*(12), 2512–2518. https://doi.org/10.1111/j.1420-9101.2009.01870.x

Westley, P. A. H., & Fleming, I. A. (2011). Landscape factors that shape a slow and persistent aquatic invasion: Brown trout in Newfoundland 1883–2010. *Diversity and Distributions*, *17*(3), 566–579. https://doi.org/10.1111/j.1472-4642.2011.00751.x

Yeates, S. E., Diamond, S. E., Einum, S., Emerson, B. C., Holt, W. V., & Gage, M. J. G. (2013). Cryptic choice of conspecific sperm controlled by the impact of ovarian fluid on sperm swimming behavior. *Evolution*, *67*(12), 3523–3536. https://doi.org/10.1111/evo.12208