

Gene expression estimates: Influence of sequencing library construction, fish sampling methods, and tissue harvesting time

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Abstract

RNA sequencing (RNA-Seq) is becoming a popular method for measuring gene expression in non-model organisms, including wild populations sampled in the field. While RNA-Seq can be used to measure gene expression variation among wild-caught individuals and can yield important biological insights into organismal function, technical variables may also influence gene expression estimates. We examined the influence of multiple technical variables on estimated gene expression in a non-model fish species, the westslope cutthroat trout (*Oncorhynchus clarkii lewisi*), using two RNA-Seq methods: 3' RNA-Seq and whole mRNA-Seq. We evaluated the effects of dip netting versus electrofishing, and of harvesting tissue immediately versus 5 minutes after euthanasia on estimated gene expression in blood, gill, muscle, and liver. We found higher RNA degradation in the liver compared to the other tissues. There were fewer expressed genes in blood compared to gill and muscle. We found no difference in gene expression among sampling methods or due to a delay in tissue collection. However, we detected fewer genes with 3' RNA-Seq than with whole mRNA-Seq and found statistically significant differences in gene expression between 3' RNA-Seq and whole mRNA-Seq. The magnitude and direction of these differences does not appear to be dependent on gene type or length. Our findings indicate that RNA-Seq is robust to the technical variables related to the field sampling techniques tested here but varies based on the tissue sampled and the RNA-Seq library used. This study advances understanding of usefulness of RNA-Seq to study gene expression variation in evolution, ecology, and conservation.

1 Introduction

RNA sequencing (RNA-Seq) is increasingly common in ecological and evolutionary studies focusing on variation in gene expression (Alvarez et al. 2014, Conesa et al. 2016, Ekblom & Galindo 2011). For example, RNA-Seq is commonly used in studies on physiology, conservation, epigenetics, and to assess organismal response to environmental variables (Todd et al. 2016, Corlett 2017, Rey et al. 2020). RNA-Seq is highly accurate for quantifying expression levels, requires less RNA sample when compared to microarrays, does not necessarily require a reference genome (e.g., Cahais et al., 2012), can uncover sequence variation in transcribed regions, and shows high reproducibility (Wang et al. 2009). However, gene expression data can be strongly influenced by biological and non-biological factors such as experimental and stochastic variation (Auer & Doerge 2010, Qian et al. 2014, Todd et al. 2016). Given the recent surge in RNA-based studies, it is critical to identify and quantify sources of variation in gene expression.

Sampling methods can be an important experimental cause of variation in estimated gene expression (Mutch et al. 2008, Passow et al. 2019). Delay in sample preservation after collection may result in higher RNA

degradation and introduce bias in estimated gene expression (e.g., Gayral et al. 2011, Romero et al. 2014). This is a consequence of mRNAs being produced in relatively short bursts in response to internal or external stimuli and having short half-lives (Ross 1995; Staton et al., 2000). Similarly, the use of different anesthetics, methods of tissue preservation, different RNA extraction methods, and timeframe between sample collection and RNA isolation can all impact RNA quality and gene expression (e.g., Debey et al. 2004, Huitink et al 2010, Jeffries et al. 2014, Mutter et al. 2004, Olsvik et al. 2007, Passow et al. 2019).

Stochastic variation in gene expression due to variation in cellular and molecular processes can result in random differences among individuals of the same population for the same genes without necessarily this being a consequence of biological (e.g., maternal effects and potentially heritable variation) or micro-environmental variation. For studies with a low count of biological replicates, this variation may be misinterpreted as biologically relevant (Hansen et al. 2011, Kaern et al. 2005). Detection of stochastic variation in gene expression may be achieved through careful sampling design (e.g., individuals vary at only one treatment) and by increasing the number of sampled individuals (Kim et al. 2015, Liu et al. 2014) to gain statistical power (Ching et al. 2014). However, often RNA-Seq experiments are limited in the number of sampled individuals due to cost, with consequent loss of statistical power and potentially misleading results (Bi & Liu 2016, Li et al. 2013).

Independent of sample size, library construction and RNA sequencing techniques may also produce variability in estimated gene expression. Whole mRNA sequencing methods often result in fragment length bias because longer transcripts are sheared into more fragments so that a higher number of reads will be assigned to them than shorter transcripts, causing an overrepresentation of larger transcripts in sequencing libraries (Ma et al. 2019, Oshlack & Wakefield 2009, Roberts et al. 2011). Cost limitations as well as fragment size bias of whole mRNA sequencing has led to the development of RNA sequencing library construction protocols that allow processing a larger number of samples in a more cost-effective manner (Meyer et al. 2011, Morrissy et al. 2009, Wu et al. 2010). The 3' RNA Tag-Seq method (also known as Quant-Seq 3' mRNA-Seq), for example, only primes the 3' poly-A tail, reducing the sequencing effort and cost, and generates an essentially uniform distribution of fragments with respect to original RNA length (Lohman et al. 2016, Ma et al. 2019).

In fish, RNA-Seq data are commonly used to investigate the effects of environmental variables (e.g. temperature, hypoxia) on gene expression (e.g., Krishnan et al. 2020, Long et al. 2015, Meyer et al. 2011, Smith et al. 2013, Wang et al. 2015). However, little is known about the influence of different sampling techniques on gene expression in fish, especially under field conditions. For example, field conditions may limit the use of optimal sampling protocols or storage methods to reduce variation (e.g., using liquid nitrogen in remote sampling locations or fast processing times for tissue isolation) (Mutter et al. 2004, Pérez-Portela & Riesgo 2013). Furthermore, field capture may also result in increased variation among individuals, including among biological replicates (Pearce et al. 2016). For example, stress related genes may be overexpressed as a result of long handling time before sampling. The impacts of handling stress on fish physiology are well understood (Sopinka et al. 2016). Although most studies focus on glucocorticoid and blood chemistry responses to capture (Milla et al. 2010, Wiseman et al. 2007, Wood et al 1983, Milligan 1996, Barton 2002, Ruane et al. 2001; see also Romero & Reed, 2005 for influence on handling time on non-fish species), gene expression responses to handling stress indicate that the magnitude, intensity, and duration of changes vary across genes, species, and tissue types (Krasnov et al. 2005, Lopez et al. 2014). While there is some evidence that a sample specimen's blood cortisol and glucose levels are affected by capture method (e.g., electrofishing), to our knowledge (Barton & Dwyer 1997, Barton & Grosh 1996, Bracewell et al. 2004), it is unknown whether gene expression is affected by capture method or handling time prior to sample collection.

Here, we test whether sampling method (electrofishing vs dip netting), processing time, and RNA-Seq libraries (3' Tag-Seq method vs. whole mRNA-Seq) influence gene expression data in multiple tissue types from westslope cutthroat trout (*Oncorhynchus clarkii lewisi*), a species of conservation concern native to western North America (Behnke 2002; Allendorf and Leary 1988; Shepard et al. 2003). The results of this study will address the sources of gene expression variation under field conditions and provide a foundation for improving future RNA-based study designs for field sampling of wild caught non-model fish and other

species.

2 Methods

2.1 Sample collection, group assignment, and tissue harvesting

All samples of westslope cutthroat trout were sampled on a single day in May 2019 at the Montana Fish, Wildlife, & Parks Sekokini Springs hatchery in West Glacier, MT. We collected 120 samples, which were divided in three treatment groups as follows (Supporting Information Table S1): group 1 = net-sampling ($n = 40$); group 2 = electrofishing ($n = 40$); group 3 = electrofishing, tissue harvested from fish 5 minutes after death by pithing (see below, tissues from group 1 and 2 fish were harvested immediately) ($n = 40$). All the fish were fry from the same breeding stock and were offspring from parents from Danaher Creek (MT). Before sampling for tissue harvesting, fish were netted as needed from the raceway and transferred to holding buckets with hatchery system water. In order to simulate field work conditions, each raceway collection was at least 10 minutes apart. Independent of the sampling method, fish were sampled five at a time to reduce the interval between sampling and tissue harvesting. Each fish was then captured by net from one of the holding buckets or shocked with a backpack electrofishing unit set to 150 volts with a standard pulse for a duration of 3 seconds and then netted. Length and weight data were collected for each fish. Fish were then euthanized by pithing and immediately processed for tissue harvesting, except for group 3. Fish from group 3 were sampled in the same way as fish from group 2, except that after euthanasia by pithing they were placed in a separate holding bucket of water for 5 minutes before tissue harvesting.

Electrofishing, which consists of a backpack mounted electrofishing unit that applies an electrical current in the water to momentarily stun the fish, is one of the most common fisheries sampling techniques. This technique may cause the fish to express genes in response to the electric current, and may affect individual fish and tissue types differently, increasing variation among biological replicates. An alternative to electrofishing is dip netting. While nets may potentially result in a lower effect on gene expression and lower risk of inadvertently killing both target and non-target organisms, it is more laborious and time consuming in the field where circumstances may not allow for long sampling periods or aquatic systems may have obstacles that prevent effective capture with nets (e.g. fallen tree limbs and rocks). Capturing fish by dip netting may still influence gene expression through stress, as the fish tries to escape being captured.

Processing times of fish and tissues may depend on which tissue needs to be harvested and how many fish are captured at the same time. Therefore, time between capture and euthanasia and duration of tissue collection were recorded for each individual. Average time in the bucket was around 2 minutes before euthanasia and average time of tissue collection was around 3 minutes (data available upon request). Tissue removal was performed using single use scalpels on a nylon cutting board. Tissue samples from each fish were collected in the following order: blood, dorsal muscle, liver, and gills. We first collected the blood immediately before euthanasia as coagulated blood may affect DNA quality (Chiari and Galtier, 2011). To obtain the blood sample, the tail was removed by a diagonal cut made through the caudal peduncle from dorsally anterior of the anal fin to ventrally posterior of the anal fin to avoid intersecting the G. I. tract. Slight pressure was applied to the body of the fish and blood was allowed to drip out of the cut directly into the 2 mL tube. Muscle tissue was sliced into smaller pieces to allow penetration of the preservative (Gayral et al. 2011). Sampling tools and the cutting board were thoroughly cleaned with 10% bleach between fish to avoid sample and tissue contamination. Tissue samples were placed in 2 mL sterile tubes filled with RNAlater (Qiagen) for preservation. Tubes were left at room temperature overnight and then stored at -80C (or in dry ice for transportation) until the RNA extraction was carried out.

2.2 RNA extraction

RNA extractions and the following laboratory procedures described below were carried out by a private company (Admera Health). The same extraction protocol was used for each of the different tissues and generally followed manufacturer instructions for QIazol (Qiagen) extraction. Briefly, up to 10 mg of tissue was mechanically homogenized in 500 μ l of QIazol. After homogenization, QIazol was added to reach 1ml and then 200 μ l of chloroform were added and mixed. For blood samples, they were centrifuged at 2000 g

for 5 minutes, the supernatant discarded and 1ml of QIAzol (Qiagen) added to the tube. Tubes were then left at room temperature for 5 minutes and vortexed a few times to ensure homogenization of the sample. 200 µl of chloroform was added and mixed. All samples (blood or other tissues, all containing 1ml of QIAzol and 200 µl of chloroform) were then incubated at room temperature for 3-5 minutes and centrifuged at 4 °C, 12,000 g for 15 minutes. The upper aqueous RNA containing phase was transferred to a new tube. An equal volume of 70% ethanol was added and mixed. The mixture was loaded into a RNeasy mini prep column (Qiagen RNeasy Mini Plus Kit) and RNA eluted following the manufacturer's protocol.

The quality of RNA was determined by Qubit HS RNA assay (ThermoFisher), and the integrity of RNA was evaluated based on RIN acquired via capillary gel electrophoresis performed using Bioanalyzer 2100 (Agilent Technologies). ANOVA was run in R using the F-test to compare RIN numbers among the samples belonging to the different groups (see below) and to compare the RIN numbers among samples belonging to different tissues in each group. These analyses were run without data from the liver for which most samples showed signs of degradation (see Results and Supporting Information Table S1).

2.4 RNA Library preparation and Sequencing

Since variation in RNA quality may affect downstream results (Passow et al. 2019), library construction and sequencing were carried out only for 81 tissue samples with a RIN value above 8.8 for 3' Tag-Seq and for 14 samples with RIN > 9.4 for whole mRNA-Seq (Supporting Information Table S1). None of these samples showed signs of RNA degradation based on the BioAnalyzer profile. Liver samples were excluded from the subsequent processing as most of the samples had a low RIN and we therefore did not have more than 3 samples per group per comparison (Supporting Information Table S1). Whole mRNA libraries (NEB) were made only for selected blood samples with similar RIN and concentration among compared groups (see below and Supporting Information Table S1 for additional information). Library preparation was performed with the NEB Ultra II RNA library prep kit with NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). For 3'-end RNA Tag-Seq, library preparation was performed with QuantSeq 3' mRNA-Seq Library Preparation Kit FWD for Illumina (Lexogen) (see Ma et al., 2019 for further details on differences between these two RNA-Seq methods). All procedures were performed according to manufacturer suggested protocols. The quantity and molecular size of the libraries were confirmed by Qubit HS DNA assay (ThermoFisher) and TapeStation 2200 system coupled with High Sensitivity D1000 ScreenTapes (Agilent). Sequencing was performed on Illumina HiSeq X with 150bp pair-end reading for all the 3' Tag-Seq samples (Lexogen) and four NEB samples, while the remaining 10 NEB samples were sequenced on a NovaSeq machine (see Results section regarding lack of difference between the NEB samples sequenced with different machines). *Raw reads will be deposited to NCBI after manuscript acceptance.*

2.5 RNASeq reads check and genome coverage

Quality checks of the raw RNA-Seq reads were performed using Fastqc (Andrews, 2014).

Reads were trimmed with trimmomatic (version 0.38, Bolger et al. 2014). Raw reads were mapped to an *Oncorhynchus mykiss* reference genome from NCBI (Omyk.1.0, https://www.ncbi.nlm.nih.gov/assembly/GCF_002163495.1/, Annotation release ID:100) using STAR (version 2.7.1a; Dobin et al., 2013) to obtain the number of genes recovered by each technique, 3' Tag-Seq vs. whole mRNA-Seq (NEB).

In order to perform the bioinformatic analyses on samples with an equal number of reads, we randomly selected 11 million reads per sample for all the analyses performed only on 3' Tag-Seq reads and 40 million reads per sample for all the analyses performed using whole mRNA-Seq (NEB) reads. Previous work has shown that >10M reads whole mRNA-Seq and 3' Tag-Seq perform similarly in recovering transcripts of different length (Ma et al., 2019). Reads were mapped again to the *Oncorhynchus mykiss* reference genome. HT-Seq (version 0.11.1; Anders et al. 2015) was then used to quantify the number of reads uniquely mapped to each gene of the *O. mykiss* reference genome. Finally, a python script provided with Stringtie (prepDE.py) was used to generate a gene counts matrix (Pertea et al., 2016).

2.6 Similarity in gene expression among samples

To assess the variation and direction of variation among samples based on their gene expression, we calculated the correlation of gene expression levels among samples and the Euclidean distances among samples in DESeq2 (version 1.22.2; Love et al., 2014) following the program directions. These measures are especially useful to assess the similarity of biological replicates (e.g., samples belonging to the same group) (Koch et al. 2018) and therefore to detect anomalies among the samples. The sample correlation matrix was calculated by performing the Pearson correlation of the normalized matrix after the variance stabilizing transformation (*vst*) was performed on the most variable 2000 genes based on the HTSeq data produced. *vst* allows taking into account the sample variability of low counts. Sample Pearson correlation is calculated in pairwise comparison among samples and ranges from -1 to 1, where a value of 0 indicates no correlation (gene expression is completely dissimilar between the two samples), while values of -1 and 1 indicate that the samples have identical expression level (-1 corresponding to negative correlation). The Euclidean distance between samples was calculated by this equation: $dist = \sqrt{1 - cor^2}$, where *cor* stands for the correlation coefficient of 2 samples. The smaller the distance, the higher the correlation among samples is. These distances were then used to build the heatmaps of sample distance of each normalized matrix, which allows the data to be shrunk towards the genes' average expression across all samples. Gene heatmaps are instead based on *vst* transformation to normalize the raw count. After this, the mean expression in each sample is then normalized to 0. Finally, differences in gene expression among the studied groups (see below) were visualized by a PCA plot using the gene count matrix after applying the variance stabilizing transformation (*vst*) to normalize the raw counts. PCA plots are useful to assess the effect of covariates and batch effects (non-biological variation due to experimental artifacts (Reese et al. 2013)).

2.7 Differential gene expression analysis

Differences in gene expression among groups were identified by differential expression analysis performed using DESeq2 on raw read counts (non-normalized, as suggested by DESeq2). To allow comparison between 3' Tag-Seq reads and whole mRNA-Seq (NEB) reads data, for the latter we only used one sequencing direction as suggested for the DESeq2 program. The false discovery rate (FDR) was adjusted to 0.05, corresponding to a recovery at most of 5% of false positives. We use the default options for all the other parameters. We look at differences in gene expression for the following comparisons between: 1. sampling methods (dip netting vs electrofishing; data analyzed with 3' Tag-Seq, NEB, and Tag-Seq vs. NEB), 2. delaying the tissue harvesting to 5 min after death or harvesting immediately (only for samples obtained by electrofishing, immediate sampling vs delayed of 5 min, data analyzed with 3' Tag-Seq, NEB, and 3' Tag-Seq vs. NEB), 3. comparisons 1 and 2 per tissue type (blood, gills, muscle; 3' Tag-Seq data), 4. 3' Tag-Seq vs NEB for all 14 samples for which we have both data independently of sampling method and tissue harvesting time, 5. tissue type within each group (sampling method or tissue harvesting time; 3' Tag-Seq data) (see Supporting Information Table S1 per information about sample size for each comparison; minimum N = 4). The log fold changes obtained from DESeq2 were used as a measure of how many more (or less) genes are expressed in one group versus the other. We considered genes having different expression if the adjusted p-value (using the adjusted p-value results in less false positives) was < 0.05.

Finally, previous work has indicated an increase in read count for longer transcripts using the whole mRNA library (NEB) than 3' Tag-Seq (Ma et al. 2019). To further address the relationship between gene length and genes differentially expressed between 3' Tag-Seq and whole mRNA library (NEB), we conducted an assessment using the known transcript length from orthologous genes in zebrafish in Ensembl 101 (Yates et al. 2020) based on gene name for genes that were recovered to be differentially expressed between the two library methods. We also used the same approach to specifically assess if transcript length could influence absence of gene expression (expression = 0) in one but not the other technique, 3' Tag-Seq or NEB.

2.8 Functional enrichment analysis

Functional enrichment analyses were carried out on genes with differential expression between tissues for a given sampling method. An over-representation analysis (ORA) was run in the WEB-based GENE SeT

AnaLysis Toolkit (WebGestalt; Liao et al. 2019) focusing on gene ontology (GO) categories associated with biological processes (BP). The gene lists of interest were genes with significantly ($p < 0.05$) greater expression in one tissue type versus another for a given sampling method. Only genes with known orthologs in zebrafish (*Danio rerio*) were used because choosing an organism of interest for a comparison is a requirement of ORA. The reference gene list for all comparisons was all zebrafish protein coding genes. Heatmaps of functionally enriched genes across sampling methods and tissue comparisons were generated using the *ggplot2* package in RStudio.

3. Results

3.1 RNA and raw sequencing data quality statistics

Out of the 120 samples for which RNA was extracted, 86 had a RIN value (a measure of RNA integrity) equal or above 8.8. Little variation in RIN scores was observed among the sampled tissues and sampling methods (Supporting Information Table S1), except for liver, which overall showed higher levels of RNA degradation and was therefore not used for library construction and sequencing. Mean and standard deviation for RIN values for the four tissues were: 9.6 ± 0.22 (blood), 9.2 ± 0.40 (muscle), 8.0 ± 1.21 (liver); 9.0 ± 1 (gill). Mean and standard deviation for RIN values for the three treatment groups without the liver were: 9.2 ± 0.43 (dip netting), 9.3 ± 0.34 (electrofishing), and 9.2 ± 1.06 (tissue harvesting after 5 minutes). We found no differences in RIN values among groups ($F = 0.299$, $df = 2$, $p = 0.74$) and in RIN values among tissues within each group ($F = 0.595$, $df = 4$, $p = 0.67$), after excluding the liver from the analyses.

RNA sequencing from 3' Tag-Seq samples regardless of tissue type yielded a total of 367.2 million reads for individuals captured by net (mean = 13.1 million \pm 0.72; $N = 28$), 328.2 million reads for samples collected by immediately after electrofishing (mean = 12.62 million \pm 0.46; $N = 26$), and 347.6 million reads from samples electrofished and processed after 5 minutes (mean = 12.87 million \pm 0.71; $N = 27$) (Supporting Information Table S1). The final number of reads per individual ranged from 11 million to 15.6 million (mean = 12.88 million \pm 0.67). On average, of the 11 million reads randomly selected for each sample, we obtained around 77% of uniquely mapped reads on the rainbow trout (*O. mykiss*) genome independently of the sampling method used (range: 67.7 - 86.3%, Supporting Information Table S1), indicating that we used good libraries (Dobin and Gingeras, 2015) for downstream analyses.

RNA sequencing from the 14 whole mRNA-Seq (NEB) samples (blood only) yielded a total of 564 million reads for individuals captured by net (mean = 112.9 million \pm 13.95; $N = 5$), 563.4 million reads for samples collected by electrofishing and sampled immediately (mean = 112.7 million \pm 22.4; $N = 5$), and 350.4 million reads from electrofishing samples processed after 5 minutes (mean = 87.6 million \pm 7.4; $N = 4$). The final number of reads per individual ranged from 77.8 to 148.8 million reads (mean = 105.6 million \pm 19.1). Number of reads per sample was therefore on average 10 times higher for NEB than 3' Tag-Seq.

After mapping the randomly selected 11 or 40 million reads of 3' Tag-Seq and NEB (see Materials and Methods) on the reference genome of *O. mykiss*, each 3' Tag-Seq and NEB sample had >8 and >28 million reads, respectively, to be used for the analyses of gene expression (Supporting Information Table S1). Reads that were uniquely mapped on the *O. mykiss* genome were similar among all the groups compared in this study (see % mapping per group above and in Supporting Information Table S1), suggesting that >10 million reads, the two RNA-Seq library constructions (3' Tag-Seq and NEB) uniquely map to roughly the same percentage of the reference genome, even if for the whole mRNA-Seq data we used 40 million reads instead of the 11 million reads used for 3' Tag-Seq.

Raw reads – i.e., before selecting 11M reads for 3' Tag-Seq and 40M reads for whole mRNA-Seq – mapped on the rainbow trout (*O. mykiss*) genome recovered a different number of genes between the two RNA library sequencing types, independently on the number of reads mapped per gene. Specifically, whole mRNA-Seq recovers two to three times more genes than 3' Tag-Seq (Supporting Information Table S1). Differential expression analysis (see below) for the 14 blood samples for which RNA libraries were built using for 3' Tag-Seq and mRNA-Seq indicates that presence/absence of genes between the two techniques is independent of gene transcript length (Supporting Information Figure S1).

3.2 Similarity in gene expression among samples

Similarity in gene expression among biological replicates - i.e., individuals belonging to the same treatment group - gives an idea of reproducibility of our data and of the overall variation among samples. Similarity in gene expression within and among groups can be estimated using the sample correlation or Euclidean distances (see Materials and Methods for further details). Pearson correlation coefficients (r) for biological replicates were above 0.9 for the majority of comparisons (same tissue and same group), with only a few pairwise comparisons having values $0.8 < r < 0.9$ (Supporting Information Table S2). Lower values near 0.8 were mostly due to one sample (blood, group 2) being different from the rest. This indicates that although variation in gene expression occurs among individuals, biological replicates are generally very similar.

Pearson r values among groups for each tissue type are slightly lower than what obtained for individuals belonging to the same group, but generally above 0.85 and with the majority of pairwise comparisons being above 0.90 (Supporting Information Table S2), indicating comparable levels of gene expression across tested groups for the same tissue. Also, in this case, the same sample mentioned above (blood, group 2) has lower r (>0.73) (Supporting Information Table S2). Pearson r values between the two sequencing platforms for whole mRNA-Seq samples (called NEB here below) are all above 0.87 except for the comparisons involving the blood sample from group 2 (>0.77) (Supporting Information Table S2), indicating that different sequencing methods did not influence the number of uniquely mapped reads. Finally, r among different tissues (for 3' Tag-Seq) and among 3' Tag-Seq vs. NEB are generally <0.5 and sometimes negative, suggesting different levels of gene expression among tissues and among the same mapped genes between the two library methods.

Heatmaps of the distance matrices for the different group comparisons provide hierarchical clustering based on sample distances. When heatmaps were made combining data from the three different tissues for 3' Tag-Seq, we found three clusters corresponding to the three different tissues (Figure 1a). However, within each cluster, as also shown by the heatmaps built with data from each tissue separately, samples belonging to different groups are clustered together, indicating no clear difference in gene expression among the tested groups (Supporting Information Figure S2). Lack of difference in gene expression among the different groups was also found using NEB data (Figure 1). Finally, comparison of 3' Tag-Seq vs NEB found differences in gene expression between the two methods; this difference was however not associated with any of the groups (Figure 1). Principal component analysis (PCA), another way to visualize variation in gene expression among samples, further supports the lack of differences among sampling methods and time of tissue harvesting and the differentiation between 3' Tag-Seq versus NEB and among the three sampled tissues (Figure 2 and Supporting Information Figure S3).

3.3 Differential gene expression

3.3.1 Dip Netting versus electrofishing sampling method

3' Tag-Seq data identified only 3 of the 39,212 genes recovered in both tested groups ($<<1\%$) with different expression across all tissues between dip netting and electrofishing sampling (adjusted p -value < 0.05). When different tissues were analyzed separately, we observed no difference in gene expression between these two sampling methods for blood samples (27,118 genes), less than 1% for gill samples (16 of 20,465 genes with adjusted p -value < 0.05), and $\sim 1.7\%$ (155 of 9,201 genes with adjusted p -value < 0.05) in muscle samples. Whole mRNA-Seq (NEB) data (available only for blood samples) confirmed the data obtained for 3' Tag-Seq with no gene showing different expression between the two sampling methods (out of 33,236 genes recovered) (Supporting Information Table S3).

3.3.2 Immediate versus delayed postmortem tissue harvesting

We found no significant difference in gene expression between samples for which tissues were harvested immediately vs. samples for which tissue was harvested ~ 5 minutes after death. For 3' Tag-Seq, when all tissues were analyzed together only one out 38,864 genes ($<<1\%$) was recovered to be significantly different with an adjusted p -value < 0.05 . Similarly to what was observed for the comparison between the two sampling techniques, when tissues were analyzed separately, blood showed no significantly differentially

expressed genes out of the 27,401 recovered, while we found $<<1\%$ differentially expressed genes for gills and muscle (18 out of 15,310 for gills and 3 out of 34,460, respectively, with adjusted p -value < 0.05) (Supporting Information Table S3). For whole mRNA-Seq (NEB), only 6 genes out of 23265 ($<<1\%$) were expressed significantly differently (adjusted p -value < 0.05 , Supporting Information Table S3).

3.3.3 3' Tag-Seq versus whole mRNA-Seq

We compared the gene expression of the same 14 blood samples for which the library for RNA-Seq was built using both 3' Tag-Seq and whole mRNA-Seq (NEB). We found 15328 out of 26316 genes (58%) showing different expression, with higher expression observed as a 2-log-fold change from the mean gene expression in one or the other library method (Supporting Information Table S3). Differentially expressed genes between 3' Tag-Seq vs. NEB were found to be 45%, 41% and 44% respectively for dip netting, electrofishing sampled immediately, and electrofishing sampled after 5 min (Supporting Information Table S3). The proportion of identical genes with differential expression favoring one of the two library methods is highly similar, although there is a slight trend toward a greater number of genes with high expression for NEB relative to 3' Tag-Seq with increased transcript length (Figure 3).

3.3.4 Comparisons between tissue type within the same sampling groups

We found that blood and muscle have the lowest number of genes recovered in both tissues compared to the other pairwise tissue comparisons (Supporting Information Tables S3 and S4). We observed differences in gene expression among tissue types (blood, muscle, gills) for each of the sampling methods. Of all the genes that are significantly differentially expressed (adjusted p -value < 0.05) among tissue types, only 25-32% are more highly expressed in blood compared to gill and muscle (Supporting Information Tables S3 and S4). Conversely, the proportion of significantly differentially expressed genes in gill and muscle was much more even and within ± 10 of 50% (Supporting Information Tables S3 and S4).

3.4 Functional enrichment

Gene ontology (GO) analysis for biological processes shows very similar enrichment of GO terms within tissue types regardless of sampling method. Among tissue types, certain GO terms are common such as RNA processing and RNA modification, which is similar to a previous fish gene expression study (Passow et al. 2018). There is also variation in the number and diversity of enriched GO terms depending on the tissue type (Supporting Information Figure S4). There are many more enriched GO terms associated with genes that are highly expressed in muscles (6 to 14 categories) compared to gills (0 to 5 categories), while the number of enriched GO categories associated with comparisons of gills (14 to 15 categories) versus blood (7 to 11 categories), and blood (9 to 11 categories) versus muscle (6 to 10 categories), are similar. Gills in particular are enriched for many GO categories related to major cell cycle processes, which fits with their active growth and turnover as epithelial cells at the aquatic interface (Stolper et al. 2019). Regardless of the sampling method, genes associated with cellular stress appear to be more highly expressed in blood and gills compared to muscle, potentially indicating tissue-specific physiological effects of the sampling process.

4 Discussion

The increasing use of RNA-Seq for ecological, physiological, and evolutionary studies on wild caught organisms has required appraisal of the influence of different sampling techniques, storage methods, processing time, and tissue types on RNA quality and data production (Camacho-Sanchez et al. 2013, Cheviron et al. 2011, Nakatsuji et al. 2019, Yu et al. 2013). Among the most important applications of RNA-Seq currently used is testing for rapid adaptation to environmental change (e.g., to captivity or climate warming), and to determine if environmentally-induced gene expression shifts are transgenerationally transmitted (e.g., Christie et al. 2016, Charlesworth et al. 2017, Skvortsova et al. 2018, Navarro-Martin et al. 2020, Savilammi et al. 2020). Our results will facilitate future research testing for transgenerational transmission of potentially epigenetic hatchery-adaptive traits in wild fish populations (e.g., Christie et al. 2016, Le Luyer et al. 2017, Wellband et al. 2020).

Evidence is accumulating regarding the effects that sampling techniques, sample processing time, RNA

degradation, and different RNA-Seq libraries have on RNA-Seq data (e.g., Gayral et al. 2012, Romero et al. 2014, Ma et al. 2019; see also Introduction). We tested these effects on samples of westslope cutthroat trout sampled using dip-netting or electrofishing. We also tested if distinct tissues may be differently affected by these conditions. Samples were sourced from a wild non-introgressed population raised in controlled environments in order to minimize variation in gene expression.

Overall, we obtained high RNA quality for all tissues (mean RIN > 9.0 for the different tissues) except liver (mean RIN = 8.0). Liver is a tissue with a high rate of protein synthesis and degradation, and the higher RNA degradation observed for this tissue in comparison to blood, muscle, and gills is likely the result of higher enzymatic activity in the liver (Carter et al. 2001, Wiseman et al. 2007). In our experiment, liver was the third tissue sampled after euthanasia, after blood and muscle, and it took us between 2 and 3 minutes to sample. Because of its importance in detoxification mechanisms, physiological studies may require sampling of this tissue. We therefore suggest sampling of liver first - if more than one tissue is sampled - to minimize RNA degradation.

We also found no difference in RNA quality among samples obtained through dip netting or electrofishing even when tissue was not harvested until 5 minutes after death. While opinions on a cutoff threshold RIN value to obtain reliable gene expression data differ, it has been shown that degraded RNA still recovers the same uniquely mapped genes as non-degraded RNA, although the coverage of mapped reads is lower for degraded RNA and gene specific (Romero et al. 2014, Wang et al. 2016). However, while RNA degradation may not strongly affect mapping, it may drastically affect estimates of differential gene expression (Chen et al. 2014, Romero et al. 2014). Furthermore, different RNA-Seq techniques may be differentially affected by RNA degradation (Adiconis et al. 2013), requiring selecting the most appropriate RNA-Seq library depending on RNA quality (Adiconis et al. 2013).

We found that gene expression among individuals belonging to the same group were generally very similar for the majority of comparisons (correlation coefficients > 0.9), independent of the sampling method or harvesting time. However, we observed among-sample variation in gene expression, reflecting the importance of larger sample size in RNA-Seq studies to decrease the influence of stochastic effects on variation in gene expression that could otherwise be interpreted as biologically relevant (Ching et al. 2020). Furthermore, we also observed similarity of expression levels among samples obtained with the two sampling methods, dip netting or electrofishing, or subjected to different tissue harvest times (immediate or 5 minutes after death). Sampling individuals of the same age, in the same environment and on the same day, with many biological replicates per treatment and using only samples with highly similar RNA quality most likely reduced the effects of non-biological variation and of non-relevant biological variation in our experiments (Fang & Cui 2010, Wong et al 2012, Yu et al. 2014).

We recovered a higher number of reads per sample with the whole mRNA-Seq library technique used here (NEB) than with 3' Tag-Seq (around 10 times higher in NEB than in 3' Tag-Seq), as expected (Ma et al. 2019). Similar to results reported by Ma et al. (2019), our recovered number of mapped genes was also higher (at least 2X higher) for samples processed with NEB than with 3' Tag-Seq, independent of the number of reads per gene and gene transcript length. This higher number suggests researchers should use whole mRNA-Seq when their research question requires genome-wide coverage of genes and study of large numbers of genes.

Selection of 11M reads and 40 M reads for 3' RNA-Tag and whole mRNA (NEB) libraries, respectively, resulted in a very similar number of unique mapped reads on the *O. mykiss* reference genome for the two library techniques (75% NEB versus 77% 3' Tag-Seq). Therefore, while RNA-Seq samples prepared using NEB libraries allow recovering more raw reads than when using the 3' Tag-Seq library, this number did not increase the proportions of uniquely mapped reads on the reference genome. Previous studies (Liu et al. 2014, Ma et al. 2019) also found similar estimates of gene expression for sequencing depth equal or above 10M reads. However, independently of the sequencing depth (in this study NEB: 40M reads and 3' Tag-Seq: 11M reads), we found different gene expression between NEB and 3' Tag-Seq, with higher estimated gene expression being gene-specific and not library-dependent. Whole mRNA-Seq has been found to detect more

differentially expressed genes, even at lower than 10M reads sequencing depth, potentially as a consequence of the increased number of mapped reads for longer transcripts for whole mRNA-Seq vs 3' RNA-Seq (Ma et al. 2019). We did find a very slight trend toward a higher proportion of genes with greater gene expression for NEB relative to 3' Tag-Seq with increasing transcript length.

Although stress levels associated with dip netting and electrofishing may differ, sampling techniques did not affect gene expression levels. This result was independent from the RNA-Seq library type (3' Tag-Seq or NEB) and tissue used. Although whole mRNA-Seq has been reported to be more sensitive to differentially expressed genes than 3' RNA-Seq methods (Ma et al. 2019), the fact that independently of the method used we found no differences in estimated gene expression between the two sampling methods further supports that researchers can confidently use either one or both of these sampling methods to obtain fish tissues for studies using RNA-Seq. As field conditions often change among sampling locations, researchers could opt to use electrofishing, where more efficient, and compare with fish obtained by netting in other localities without worrying about introducing extraneous variation in gene expression.

We also found that harvesting the tissue immediately or 5 minutes after death did not produce variation in gene expression, suggesting that it is safe to euthanize fish in batches and then proceed to tissue harvesting. In our work, the maximum processing time of the last tissue harvested after death was approximately 10 min (for fish processed 5 minutes after death). Although sampling techniques and tissue processing time did not influence variation in gene expression, we observed a large proportion of differentially expressed genes among the different tissues.

We found fewer expressed genes in blood compared to gill and muscle, and a smaller proportion of genes with higher expression in blood than in the other two tissues. Blood and muscle were also the tissues with the least number of expressed genes in common. Gill was the tissue in which the higher number of total expressed genes was recovered. This may be due to the active cellular processes occurring in gills (Stolper et al. 2019) - especially in animals that are experiencing growth as were the ones sampled by us - as supported by our finding on the type of genes found to be highly expressed in this tissue (e.g., gene related to metabolic and growth-related processes). Depending on the study question, sampling different tissues may ensure that multiple genes and multiple biological processes are considered for studies on differential gene expression.

In summary, our study indicates that differential gene expression results are likely to be comparable for dip netting and electrofishing. Additionally, gill, blood, and muscle all produce good quality RNA with reliable results if sampled within 10 minutes from death. Only liver samples showed reduced quality results. Finally, although whole mRNA-Seq detects more differentially expressed genes, this did not produce different results in terms of distinct gene expression among the groups tested here. 3' Tag-Seq can therefore be more cost effective, ensuring a sufficient depth coverage and allowing processing larger samples sizes at a lower cost, thus potentially increasing statistical power of detection of differential gene expression. Consequently, depending on the study question, sequencing a large number of individuals using 3' Tag-Seq (and a subset of samples with whole mRNA-Seq) will often be the best strategy to test for differences in gene expression among tested groups. Our study provides data crucially-needed to advance use of RNA-Seq to investigate gene expression variation and its role in phenomena such as adaptation to environmental variation and climate change in natural populations

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Data Accessibility Statement

Raw reads produced for this work are uploaded to NCBI and will be available after manuscript acceptance.

Author contribution

MB, MK, GL, and YC designed the study. LH, SR, JD and GL performed the sampling. Analyses were performed by SG, YC, and commissioned to Admera Health. NM, LH, SG, and YC wrote the manuscript. GL and YC provided funding for this project. All authors have edited and approved the manuscript.

Figure Legends

Figure 1: Sample-to-sample distance heatmap . Sample-to-sample distance heatmaps for the comparison between different sampling techniques, different tissue harvesting time, and different mRNA-Seq libraries. The rows and columns are arranged based on hierarchical clustering, so that samples with similar expression profiles are positioned near to each other. The color scale represents the distance between samples. A value of distance 0 indicates that two samples have identical gene expression. The smaller the distance is, the higher is the correlation between two samples. Treatment groups (called “*condition*”) compared are indicated in different colors next to each heatmap. Condition 1 = fish captured by dip netting, condition 2 = fish captured by electrofishing processed immediately, condition 3 = fish captured by electrofishing processed 5 minutes after euthanasia. **A.** 3’ Tag-Seq dip netting versus electrofishing for all tissues combined, **B.** 3’ Tag-Seq electrofishing with immediate sampling versus electrofishing with delayed sampling for all tissues combined, **C.** NEB dip netting versus electrofishing only for blood samples, **D.** NEB electrofishing with immediate sampling versus electrofishing with delayed sampling only for blood samples, **E.** NEB versus 3’ Tag-Seq comparisons for dip netting versus electrofishing only for blood samples, **F.** NEB versus 3’ Tag-Seq comparisons for electrofishing with immediate sampling versus electrofishing with delayed sampling only for blood samples.

Figure 2: PCA plots showing PC1 and PC2 for samples that are differentially expressed among sampling techniques, tissue harvesting time, and library preparation methods. Treatment groups (called “*condition*”) compared are indicated in different colored symbols next to each PCA plot. Condition 1 = fish captured by net, condition 2 = fish captured by electrofishing processed immediately, condition 3 = fish captured by electrofishing processed 5 minutes after euthanasia. **A.** 3’ Tag-Seq dip netting versus electrofishing for all tissues combined, **B.** 3’ Tag-Seq electrofishing with immediate sampling versus electrofishing with delayed sampling for all tissues combined, **C.** NEB dip netting versus electrofishing only for blood samples, **D.** NEB electrofishing with immediate sampling versus electrofishing with delayed sampling only for blood samples, **E.** NEB versus 3’ Tag-Seq comparisons for dip netting versus electrofishing only for blood samples, **F.** NEB versus 3’ Tag-Seq comparisons for electrofishing with immediate sampling versus electrofishing with delayed sampling only for blood samples.

Figure 3. Violin and box plots comparing gene expression versus gene length for the whole mRNA-Seq (NEB) and 3’ Tag-Seq library methods. Each individual plot compares the number of genes with significantly different base mean expression for NEB versus 3’ Tag-Seq, calculated as $\log_{\text{base}} \text{mean NEB} - \log_{\text{base}} \text{mean 3’ Tag-Seq}$. Genes with equal expression fall on the zero line of the y-axis; genes with higher expression for the whole mRNA transcriptome versus 3’ Tag-Seq have positive numeric values above 0, while genes with higher expression for 3’ Tag-Seq vs whole mRNA transcriptome have negative numeric values below 0.

Supporting Information Legends:

Table S1: Sample, RNA quality, gene counts, and library information. Sheet “*Samples All*” lists all samples collected (sample ID and Admera Health ID for 3’ Tag-Seq and for the whole mRNA-Seq, NEB) with information about the treatment group they belong to, tissue type, sampling method, RIN value, and RNA concentration. Sample size used for each comparison and divided for tissue type, treatment group, and library preparation is also indicated. Sheet “*3’ RNA Tag Seq*” lists all samples used for the 3’ Tag-Seq library with the following information for each sample: treatment group, sample ID, Admera Health ID’s, tissue type, sampling method, RIN value, concentration, raw read count, read count after mapping

the randomly selected 11 million reads, and percentage of uniquely mapped genes on the reference genome. Sheet “*Whole mRNA Seq*” lists all samples used in the whole mRNA-Seq library detailing for each sample the following: treatment group, sample ID, Admera Health ID’s (and new Admera Health ID if existing), tissue type, sampling method, RIN value, concentration, raw read count (PE and single), read count after mapping the randomly selected 40 million reads, and percentage of uniquely mapped genes on the reference genome. Sheet “*Gene count*” lists all the 14 samples processed with both 3’ Tag-Seq and whole mRNA-Seq detailing for each sample the library type, IDs, gene count (using different number of reads per each gene as a filter), and percentage of uniquely mapped genes on the reference genome.

Table S2: Correlation coefficient values between samples for all comparisons. Comparisons among different treatment groups and tissues are indicated on separate sheets. Each sheet has an Admera Health sample ID, treatment group of belonging; depending on the comparisons information about library type (3’ Tag-Seq or whole mRNA-Seq NEB), sequencing platform, and tissue type are also provided.

Table S3: Output results of the Differential Expression Analysis . Results of Differential Expression Analysis done with DESeq2 for all comparisons, each of them presented on a separate sheet.

Table S4: Summary of gene expression patterns for different sampling methods and tissue types. The total numbers of genes with detectable expression for each sampling/tissue comparison are indicated along with the number and proportion of genes with significantly higher gene expression in one of the two tissues being compared for each sampling method.

Figure S1: Bar plot of transcript length versus number of non-expressed genes for each RNA-Seq library technique. In the legend next to the Figure, Full corresponds to whole mRNA-Seq and tag corresponds to 3’ Tag-Seq. Data based on the 14 samples sequenced using both techniques.

Figure S2: Sample-to-sample distance heatmap . Sample-to-sample distance heatmaps for the comparison between different sampling techniques and different tissue harvesting time for the different tissues. The rows and columns are arranged based on hierarchical clustering, so that samples with similar expression profiles are positioned near to each other. The color scale represents the distance between samples. A value of distance 0 indicates that two samples have identical gene expression. The smaller the distance is, the higher is the correlation between two samples. Treatment groups compared (called “*condition*”) are indicated in different colors next to each heatmap. Condition 1 = fish captured by dip netting, Condition 2 = fish captured by electrofishing processed immediately, Condition 3 = fish captured by electrofishing processed 5 minutes after euthanasia. **A.** 3’ Tag-Seq dip netting versus electrofishing only for blood samples, **B.** 3’ Tag-Seq dip netting versus electrofishing only for gill samples, **C.** 3’ Tag-Seq dip netting versus electrofishing only for muscle samples, **D.** 3’ Tag-Seq electrofishing with immediate sampling versus electrofishing with delayed sampling only for blood samples, **E.** 3’ Tag-Seq electrofishing with immediate sampling versus electrofishing with delayed sampling only for gill samples, **F.** 3’ Tag-Seq electrofishing with immediate sampling versus electrofishing with delayed sampling only for muscle samples.

Figure S3: PCA plots showing PC1 and PC2 for samples that are differentially expressed among sampling techniques and tissue harvesting time for the different tissues. **A.** 3’ Tag-Seq dip netting versus electrofishing only for blood samples, **B.** 3’ Tag-Seq dip netting versus electrofishing only for gill samples, **C.** 3’ Tag-Seq dip netting versus electrofishing only for muscle samples, **D.** 3’ Tag-Seq electrofishing with immediate sampling versus electrofishing with delayed sampling only for blood samples, **E.** 3’ Tag-Seq electrofishing with immediate sampling versus electrofishing with delayed sampling only for gill samples, **F.** 3’ Tag-Seq electrofishing with immediate sampling versus electrofishing with delayed sampling only for muscle samples.

Figure S4: Gene ontology (GO) heatmap based on over-representation analysis (ORA) of genes with greater tissue-specific expression. Rows represent GO descriptions and IDs for biological processes. Columns represent the sampling method [dip netting (N), electrofishing with rapid sampling (E), electrofishing with 5-minute wait time (E5)], followed by the tissue with significantly higher gene expression for listed GO terms, followed by the tissue with significantly lower gene expression. Heatmap intensity is a

function of the false detection rate (FDR) of enriched GO terms.



