Influence of RNA-Seq library construction, sampling methods, and tissue harvesting time on gene expression estimation

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Abstract

RNA sequencing (RNA-Seq) is a popular method for measuring gene expression in non-model organisms, including wild populations. While RNA-Seq can measure gene expression variation among wild-caught individuals and can yield important biological insights into organism function, sampling methods may also influence gene expression estimates. We examined the influence of multiple technical variables on estimated gene expression in a non-model fish, the westslope cutthroat trout (Oncorhynchus clarkii lewisi), using two RNA-Seq library types: 3' RNA-Seq and whole mRNA-Seq. We evaluated effects of dip netting versus electrofishing, and of harvesting tissue immediately versus 5 minutes after euthanasia on estimated gene expression in blood, gill, and muscle. We detected 30% more genes with whole mRNA-Seq than with 3' RNA-Seq and found that 58% of genes were significantly differently expressed between 3' RNA-Seq and whole mRNA-Seq. Our findings indicate that 3' RNA-Seq and whole mRNA-Seq are robust to the technical variables related to the field sampling approaches tested here with a lack of differential gene expression among sampling methods and tissue collection time after euthanasia. However, we found that gene expression varied based on which RNA-Seq library type was used on the same set of samples. Our study suggests researchers could safely rely on different fish sampling strategies in the field and save money and analyze more individuals using 3' RNA-Seq, but should use whole mRNA-Seq when working with a species without good genomic resources, and when maximizing the number of genes identified and detecting alternative splicing are important.

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), It has been used in research on physiology, conservation, 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 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 therefore critical to identify and quantify non-biological sources of variation in gene expression estimates.

Tissue 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, for example by

increasing storage time in buffer at room temperature for more than 10 days, 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 or rapid 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).

Variation in gene expression due to stochastic variation in cellular and molecular processes can result in random differences among individuals of the same population for the same genes without necessarily being a consequence of micro-environmental variation or other biological factors (e.g., maternal effects and potentially heritable variation). For studies with few 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, RNA-Seq experiments are often 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).

Higher sequencing cost has led to the development of RNA library construction protocols that allow processing and sequencing a larger number of samples in a more cost-effective manner (Meyer et al., 2011; Morrissy et al., 2009; Wu et al., 2010). 3' RNA-Seq methods only primes the 3' poly-A tail, thus reducing the sequencing effort and cost (Lohman et al., 2016; Ma et al., 2019). Independent of sample size, library construction and RNA sequencing techniques however may also produce variability in detection of transcripts, detection of differentially expressed genes among treatments and observed differences in gene expression between whole mRNA-Seq and 3' RNA-Seq (e.g., Crow et al., 2020; Jarvis et al.; 2020; Ma et al., 2019; Tandonnet & Torres, 2017). Furthermore, whole mRNA libraries and 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 (Ma et al., 2019; Oshlack & Wakefield, 2009; Roberts et al., 2011). On the other hand, 3' RNA-Seq generates an essentially uniform distribution of fragments with respect to original RNA length (Lohman et al., 2016; Ma et al., 2019). Although there are methods to correct for the bias in gene expression due to differences in transcript length, the detection and sampling of transcripts is still higher – especially for longer transcripts – when using classical mRNA-Seq approaches (Crow et al., 2022; Ma et al., 2019; Mandelboum et al., 2019; Tandonnet & Torres, 2017). Finally, whole mRNA-Seq libraries permits identification of alternative splicing at a single gene, as library and sequencing with this method capture different fragments and transcripts for the same locus (Crow et al., 2022).

In many species including 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; Jeffries et al., 2021). However, there is little known about the influence of different methods used to sample individuals , under field conditions on gene expression. Field conditions may limit the use of optimal sampling protocols or storage methods(Mutter et al., 2004; Pérez-Portela & Riesgo, 2013). Handling time of individuals before tissue sampling may also be longer than in the lab and affect gene expression differently depending on the field sampling technique and tissue used.

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 following 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 of 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 evidence that 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 unclear 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' RNA-Seq – here called QuantSeq - vs. whole mRNA-Seq – here called NEB) 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). 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 methods. This method 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 and less effective 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 capture.

The results of this study will 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 collected on a single day in May 2019 at the Montana Fish, Wildlife, & Parks Sekokini Springs hatchery in West Glacier, MT (USA). We collected 30 fish divided in three treatment groups (10 fish per group), each with 4 tissues for a total of 120 samples as follows (Tables 1 and 2 and available on Dryad): group 1 = net-sampling with immediate tissue harvest (n samples = 40); group 2 = electrofishing with immediate tissue harvest (n samples = 40); group 3 = electrofishing, tissue harvested from fish 5 minutes after death by pithing (see below, n samples = 40). All fish were fry (1 year old; fish were non-sexually mature as they cannot be sexed until 2-4 years old) from the same breeding stock and were offspring (F1) from wild parents from Danaher Creek (MT). Average size of fish was 108 mm +/- 11 and average weight was 10g +/- 3.

Fish were gently netted five at the time from the raceway into holding buckets containing hatchery system water. Fish were then either captured from the bucket by net or electrofished backpack electrofishing unit set to 150 volts with a standard pulse for a duration of 3 seconds. Fish were then euthanized by pithing and 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 pithing they were placed in a separate holding bucket of water for 5 minutes before tissue harvesting to test for the influence of delayed tissue harvesting. Time between capture and euthanasia and duration of tissue collection were recorded for each individual. Average time in the bucket was approximately 2 minutes before euthanasia and average time of tissue collection after pithing was approximately 3 minutes, except for group 3, for which tissue harvesting began 2-3 min after the 5 min from pithing. Length and weight data were collected for each fish. Sample information, including times of tissue harvest after euthanasia for each sample can be found in Table S1 on Dryad).

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 RNA 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 gastrointestinal 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 first

and then purified water 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. All sampling was carried out according to the Institutional Animal Care and Use Committee (IACUC) approved permit #AUP 007-19GLFLBS-062819 to GL.

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 µl of QIazol. After homogenization, QIazol was added to reach 1ml and then 200 µl 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 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 (RNA integrity number, varies between 1 – 10 with 10 when there is no degradation) 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 (average RIN 8.0±1.21 , see also data on Dryad). All the results presented in the Result section therefore do not include data on the liver samples.

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 for 81 tissue samples with a RIN value above 8.8 for QuantSeq and a subset of 14 blood samples (for which we also had QuantSeq data) with RIN> 9.4 for NEB (Tables 1 and 2 and data on Dryad). None of these samples showed signs of RNA degradation based on the BioAnalyzer profile(Tables 1 and 2 and data on Dryad). Whole mRNA libraries (NEB) were made only for 14 selected blood samples with similar RIN and concentration among compared groups (Tables 1 and 2). 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). 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 QuantSeq 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 were deposited on NCBI (SRA PRJNA691889, available 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 using the default parameters (version 0.38, Bolger et al. 2014). Raw reads were mapped to an*Oncorhynchus mykiss* reference genome from NCBI (Omyk_1.0, htt-

ps://www.ncbi.nlm.nih.gov/assembly/GCF_002163495.1/, Annotation release ID:100) using STAR (version 2.7.1a; Dobin et al., 2013; Dobin and Gingeras, 2015) to obtain the number of genes detected by each technique, QuantSeq vs. NEB.

In order to perform bioinformatic analyses on samples with an equal number of uniquely mapped reads among the two library types (as also previously done by others, see Ma et al., 2019), we randomly selected 11 million and 40 million reads per sample for all analyses performed on QuantSeq and NEB, respectively. Previous work has shown that using ˜ >10M reads does not increase the amount of uniquely mapped reads, after which the ability to detect differently expressed genes becomes independent of sequencing depth (Ramsköld, 2009; Liu et al. 2014; Ma et al., 2019; see also Crow et al., 2022 for an in depth discussion on the issue of redundancy of reads). Transcripts are randomly sheared into fragments with NEB but not QuantSeq. Consequently, the number of reads with NEB are proportional to the number of fragments not transcripts, whereas the number of reads with QuantSeq is proportional to the number of transcripts. Because of this, more reads may be needed for NEB than for QuantSeq to have a similar percentage of uniquely mapped reads. However, having many fragments corresponding to the same transcript is redundant and not useful for gene expression quantification (Crow et al., 2022). At the same time, especially for longer transcripts, the increased number of fragments per transcript obtained with NEB library increases transcript detection (Crow et al., 2022; Ma et al., 2019). The issue for whole mRNA-Seq of over-counting the same transcript due to multiple fragments corresponding to the same locus is especially relevant at higher sequencing depths (see Crow et al., 2022 for a detailed discussion on this issue), so that randomly selecting a lower number of reads to obtain an equal number of uniquely mapped reads among the two library types overcome this issue (see also Crow et al., 2022).

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 DESeq 2 (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 between 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 indicate that the samples have identical expression level (and -1 corresponds to negative correlation). The Euclidean distance between sample expression profiles 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 between samples. These distances were then used to build the heatmaps of sample distance of each normalized matrix, which allows the data to be shrunken towards the genes' average expression across all samples. Gene heatmaps were 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 QuantSeq reads and NEB reads data, we only used one sequencing direction as suggested for the DESeq2 program for the latter. The false discovery rate (FDR) was adjusted to 0.05, corresponding to a recovery at most of 5% of false positives following the DESeq2 manual. We use the default options for all the other parameters. We look at differences in gene expression between sampling methods, harvest tissue time, tissue type, and QuantSeq vs. NEB in Table 2: (see Tables 1, 2 and data on Dryad per detailed information about comparisons and sample size for each comparison; minimum N = 4). The log2 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 NEB than QuantSeq (Ma et al. 2019; but see Crow et al., 2022). To further address the relationship between gene length and genes differentially expressed between QuantSeq and 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 detected to be differentially expressed between the two library types. We used the zebrafish instead of the rainbow trout genome as the former has a more curated annotation (and thus more precise gene length information) than the latter. We also used the same approach to specifically assess if transcript length could influence absence of gene expression or not detection (mean base = 0 in DESeq2 output) in one but not the other library type, QuantSeq or NEB.

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 (Table 1 and data on Dryad). Mean and standard deviation for RIN values for the three tissues were: 9.6±0.22 (blood), 9.2±0.40 (muscle), 9.0±1 (gill). Mean and standard deviation for RIN values for the three treatment groups: 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 ($F = 0.595$, $df = 4$,$p = 0.67$).

Table S1 on Dryad). The final number of reads per individual for QuantSeq libraries ranged from 11 million to 15.6 million (mean = 12.88 million ± 0.67; data on Dryad). 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%, Table S1 on Dryad .

RNA sequencing from the 14 NEB samples (blood only) yielded a total of 564 million reads for individuals captured by net (mean = 112.9 million+ 13.95; N = 5), 563.4 million reads for samples collected by electrofishing and sampled immediately (mean = 112.7 million +22.4; N = 5), and 350.4 million reads from electrofishing samples processed after 5 minutes (mean = 87.6 million + 7.4; N = 4). The final number of reads per individual ranged from 77.8 to 148.8 million reads (mean = 105.6 million +- 19.1). On average, of the 40 million reads randomly selected for each sample, we obtained on average 75% of uniquely mapped reads on the *O. mykiss* genome (Table S1 on Dryad).

Mapping reads on the rainbow trout (*O. mykiss)* genome and looking at the output of DESeq2 between the two library types and for the same samples, we found that NEB detected 30% more genes than QuantSeq (Table S2 on Dryad). A gene was considered to be detected/expressed when basemean was different from 0. Specifically, for the 14 blood samples for which the two different types of RNA-Seq libraries were built, we found that NEB and QuantSeq detected approximately 35K and 26K genes, respectively, which mapped onto the annotated rainbow trout (*O. mykiss*) genome. 25K genes were detected by both library types. However, 9K genes were detected by NEB but not QuantSeq, and 1K genes were detected by QuantSeq but not NEB. Presence/absence of genes detected by one or the other library type is independent of gene

transcript length (Figure 1).

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 equal or above 0.9 for 97% of comparisons (same tissue and some tissue within a group) (Table S3 on Dryad). This indicates that although variation in gene expression occurs among individuals, biological replicates are generally very similar.

Pearson r values between the two sequencing platforms for NEB are all above 0.9 for the samples belonging to the same group (Supporting Information Table S2), indicating that different sequencing methods did not influence the number of uniquely mapped reads. Finally, r among different tissues (for QuantSeq) and among QuantSeq 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 types.

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 QuantSeq, we found three clusters corresponding to the three different tissues (Figure 2A). 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 S1). Lack of difference in gene expression among the different groups was also found using NEB data (Figure 2).

Finally, comparison of QuantSeq vs. NEB found differences in gene expression between the two methods; however, this difference was not associated with any of the groups (Figure 2). 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 QuantSeq versus NEB and among the three sampled tissues (Figures 3 and 4 and Supporting Information Figure S2).

3.3 Differential gene expression

3.3.1 Dip Netting versus electrofishing sampling method

QuantSeq data identified only 3 out of 39,212 genes (0.008%) that were significantly (adjusted p- value < 0.05) differentially expressed between dip netting and electrofishing across all tissue types. When gene expression between dip netting and electrofishing was analyzed separately for different tissue types, 0 out of 27,118 genes were differentially expressed in blood, 16 out of 20,465 genes (0.08%) in gills, and 155 out of 9,201 genes (1.7%) in muscle. NEB data (available for blood samples only) confirmed QuantSeq data, with no genes showing differential expression between the two sampling methods (0 out of 33,236 genes) (Table S2 on Dryad).

3.3.2 Immediate versus delayed postmortem tissue harvesting

We found no significant difference (adjusted p- value < 0.05) in gene expression between samples for which tissues were harvested immediately versus ˜5 minutes after euthanasia. QuantSeq data identified only 1 out of 38,864 genes (0.003%) that were significantly differentially expressed between tissue harvesting times across all tissue types. Similar to what was observed between sampling techniques, when tissues were analyzed separately for harvesting times, 0 out of 27,401 genes were differentially expressed in blood, 18 out of 15,310 genes (0.1%) in gills, and 3 out of 34,460 genes (0.009%) in muscle (Table S2 on Dryad). NEB data (available for blood samples only) was similar to QuantSeq data, with only 6 out of 23,265 genes (0.03%) showing differential expression between the two sampling methods (Table S2 on Dryad).

3.3.3 QuantSeq versus whole mRNA-Seq

We compared the gene expression of the same 14 blood samples (Ntot=28) among RNA-Seq libraries built using QuantSeq and NEB. We found 15,328 out of 26,316 genes (58%) that were significantly (adjusted$p$ -value <0.05) differentially expressed among library types (Table S2 on Dryad). Specifically, we found 9852 (64%) transcripts with higher expression in NEB versus QuantSeq and 5476 (36%) transcripts with higher expression in QuantSeq versus NEB. Within sampling methods, the percentage of differentially expressed genes between QuantSeq and NEB was 45%, 41%, and 44% for dip netting, electrofishing sampled immediately, and electrofishing sampled after 5 min, respectively (Table S2 on Dryad). The proportion of identical genes with differential expression favoring one of the two library methods is very similar, although does favor NEB with increased transcript length (Figure 5). Log of the basemean expression differences for NEB relative to QuantSeq was only 0.098 for transcripts <1000 bp, but was 0.27 for transcripts between 5000-6000 bp (Figure 5).

3.3.4 Comparisons between tissue type within the same sampling groups

We found that blood and muscle have the lowest number of genes with detected expression in both tissues compared to the other pairwise tissue comparisons (Tables S2 and S4 on Dryad). 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 (Tables S2 and S4 on Dryad). Conversely, the proportion of significantly differentially expressed genes in gill and muscle was much more even and within +-10 of 50% (Tables S2 and S4 on Dryad).

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 is testing for rapid adaptation to environmental change (e.g., Narum & Campbell, 2015; Connon et al., 2018) and for its inheritance (e.g., Christie et al. 2016, Charlesworth et al. 2017, Skvortsova et al. 2018, Navarro-Martin et al. 2020, Savilammi et al. 2020), and for addressing questions in evo-devo (e.g., Roux et al., 2015; Liu et al., 2020).

In our work, we tested if different sampling techniques influenced gene expression across different tissues in westslope cutthroat trout. Overall, we obtained high RNA quality for all tissues (mean RIN> 9.0 for the different tissues) except liver (mean RIN = 8.0). Liver has 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 target 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 euthanasia. While opinions on a cutoff threshold RIN value to obtain reliable gene expression data differ, it has been shown that partially degraded RNA may still detect the same uniquely mapped genes as non-degraded RNA, although the coverage of mapped reads is lower for partially 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 and tissue type were 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 euthanasia). 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 detected a higher number of mapped and expressed genes (˜30% higher) for samples processed with NEB than with QuantSeq, independent of gene transcript length. Others have proposed traditional whole mRNA to detect more genes than 3' RNA-Seq libraries (e.g., Crow et al., 2022; Ma et al., 2019; Xiong et al., 2017). Furthermore, we observed that while NEB detected 30% more expressed genes than QuantSeq, QuantSeq also detected a smaller number of expressed genes that were not found with NEB. Finally, for the same samples processed with both library types, we found different gene expression between NEB and QuantSeq, with a higher proportion of genes (58%) with greater gene expression for NEB relative to QuantSeq. As we did not find differentially expressed genes between sampling techniques and processing time after euthanasia (see below), we could not estimate if NEB and QuantSeq would detect a different number of differentially expressed genes, as previously reported by others (e.g., Crow et al., 2022; Ma et al., 2019; Tandonnet and Torres, 2017). Different detection of genes and differentially expressed genes between the two library types has been proposed to depend on the length of the transcript and how accurate and complete the annotation of the genome of the organism is. In general, QuantSeq seems to perform better in detecting shorter transcripts and whole mRNA-Seq in detecting longer ones. Furthermore, since QuantSeq library data rely on mapping the reads to the 3' UTR on the species' genome to detect genes, and since UTR regions are generally more variable in the genome than protein coding regions, better annotated and complete genomes facilitate mapping and detection of transcripts/genes. In this study, mapping was carried out on a closely related salmonid species, since the genome of the westslope cutthroat trout is currently not available, and this can explain why many more expressed genes were detected with NEB than QuantSeq. The higher number of detected expressed genes suggests that researchers should use whole mRNA-Seq for work on species with limited genomic resources (Crow et al., 2022). Furthermore, as QuantSeq libraries only allow amplification of the 3' end of the transcript, different transcripts resulting from alternative cleavage sites and splicing would only be detected if the 3' UTR were different. Traditional whole mRNA-Seq should therefore be preferred if identifying distinct spliced transcripts may be of interest for the study question. Finally, others (Crow et al., 2022) have reported how the increased sequencing depth of traditional whole mRNA-Seq methods may produce redundancy of reads without increasing the power of detection of differentially expressed genes. As one of the major advantages that have been reported for QuantSeq (and 3' RNA-Seq in general) is the reduced sequencing cost, a reduced sequencing depth for whole mRNA-Seq library can produce the desired data at a reduced cost.

One of the goals of this study was to test if different sampling methods and processing time would affect gene expression. Although stress levels associated with dip netting and electrofishing may differ, we found that sampling technique did not affect gene expression levels. This result was independent of the RNA-Seq library type (QuantSeq 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), independent of the RNA-Seq library used, we found no difference in estimated gene expression between the two field collection methods. 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 introducing extraneous variation in gene expression.

We also found that harvesting the tissue immediately or 5 minutes after euthanasia 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 euthanasia was approximately

10 min (for fish processed 5 minutes after euthanasia). 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 fewest expressed genes in common. Gill tissue had the highest number of detected expressed genes. This may be due to active cellular processes occurring in gills further amplified in actively growing animals (Stolper et al. 2019). 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 5-10 minutes from euthanasia. Only liver samples showed RNA degradation. Finally, although NEB library detected more expressed genes, this did not lead to different results in terms of distinct gene expression among the groups tested here. If detecting alternative splicing is not of interest for the study question and if working with an organism with good genomic resources (available genome or a good genome available for a closely related species), researchers can rely on using either of the library types tested here, QuantSeq or NEB and sequence them at similar depth, reducing the cost of NEB library sequencing. However, when it is crucial to detect as many genes as possible and when working with an organism lacking good genomic resources, whole RNA-Seq is recommended. These findings advance the 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 (SRA PRJNA691889) and will be available after manuscript acceptance. All the supplementary tables mentioned in this study have been deposited on Dryad and can be found here: https://datadryad.org/stash/share/0e5VwzuQzjim9fzQppIl8Q6g7eVqaX2NlaQ22kGetX4

Data Citation

Raw reads and supplementary tables and data. Chiari, Y., Howard, L., Moreno, N., Relyea, S., Dunnigan, J., Boyer, M.C., Kardos, M., Glaberman, S., Luikart, G. (2022) RNA-Seq reads and analysis outputs. NCBI (SRA PRJNA691889) and Dryad

Legend for the file deposited on Dryad

Table S1: Sample, RNA quality, gene counts, and library information. Sheet "*Samples All* " lists all samples collected (sample ID and Admera Health ID for QuantSeq and for NEB) with information about the treatment group they belong to, tissue type, sampling method, length and weight of the fish, 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 "*QuantSeq* " lists all samples used for the QuantSeq 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 "*NEB* " lists all samples used in the NEB 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.

Table S2: 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. Groups 1, 2, and 3 refer to sampling by netting, electrofishing, and electrofishing with processing 5 min after euthanasia, respectively.

Table S3: 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.

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.

Author contribution

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

Table 1: Samples and RNA quality. List of all the samples on which transcriptomic data were obtained in this study (sample ID and Admera Health ID for QuantSeq and NEB) with information about the treatment group they belong to, tissue type, sampling method, RIN value, and RNA concentration (ng/ul). Full list of samples and relative information for which RNA was extracted can be found in Table S1 on Dryad

Sample ID	QuantSeq	NEB ID	Group #	Tissue	Sampling Method	RIN	Concentra
1_1_1	19101XR-01-41		1	blood	dip net	9	60.3
1_1_3	19101XR-01-42		1	muscle	dip net	9.2	188.5
1_1_5	19101XR-01-44		1	gill	dip net	9.3	68.9
1_10_1	19101XR-01-77	19101XR-01-77NEB	1	blood	dip net	9.8	166.1
1_10_3	19101XR-01-78		1	muscle	dip net	8.9	586.1
1_10_5	19101XR-01-80		1	gill	dip net	9.2	784
1_2_1	19101XR-01-45		1	blood	dip net	9.4	51.8
1_2_3	19101XR-01-46		1	muscle	dip net	10	150.7
1_2_5	19101XR-01-48		1	gill	dip net	9.3	361.9
1_3_1	19101XR-01-49		1	blood	dip net	9.2	75.3
1_3_3	19101XR-01-50		1	muscle	dip net	9.6	307.3
1_3_5	19101XR-01-52		1	gill	dip net	9.5	69.3
1_4_1	19101XR-01-53	19101XR-01-53NEB	1	blood	dip net	9.5	80.8
1_4_3	19101XR-01-54		1	muscle	dip net	8.9	224.4
1_4_5	19101XR-01-56		1	gill	dip net	9.2	201.8
1_5_1	19101XR-01-57	19101XR-01-57NEB	1	blood	dip net	9.6	195.2
1_5_3	19101XR-01-58		1	muscle	dip net	9	90.7
1_5_5	19101XR-01-60		1	gill	dip net	8.8	44.5
1_6_1	19101XR-01-61	19101XR-01-61NEB	1	blood	dip net	9.6	64.8
1_6_3	19101XR-01-62		1	muscle	dip net	8.8	122.5
1_6_5	19101XR-01-64		1	gill	dip net	9.2	91
1_7_1	19101XR-01-65		1	blood	dip net	9.4	88
1_7_3	19101XR-01-66		1	muscle	dip net	9.4	248.8
1_8_1	19101XR-01-69	19101XR-01-69NEB	1	blood	dip net	9.4	154.3
1_8_3	19101XR-01-70		1	muscle	dip net	8.8	128.5
1_8_5	19101XR-01-72		1	gill	dip net	9.2	575.4
1_9_1	19101XR-01-73		1	blood	dip net	9.7	40.5
1_9_5	19101XR-01-76		1	gill	dip net	9.3	483.3
3_1_1	19101XR-01-121	19101XR-01-121NEB	2	blood	efishing	9.6	105.5
3_1_3	19101XR-01-122		2	muscle	efishing	8.8	384.8
3_1_5	19101XR-01-124		2	gill	efishing	9.5	489.9
3_10_1	19101XR-01-157	19101XR-01-157NEB	2	blood	efishing	9.8	92.7
3_10_3	19101XR-01-158		2	muscle	efishing	9.4	234.3
3_10_5	19101XR-01-160		2	gill	efishing	9.1	526.7
3_2_3	19101XR-01-126		2	muscle	efishing	9.1	388.6
3_2_5	19101XR-01-128		2	gill	efishing	9.2	325
3_3_3	19101XR-01-130		2	muscle	efishing	9.3	241.3
3_3_5	19101XR-01-132		2	gill	efishing	9.2	124.6
3_4_1	19101XR-01-133	19101XR-01-133NEB	2	blood	efishing	9.6	151.3
3_4_3	19101XR-01-134		2	muscle	efishing	8.8	294.2
3_4_5	19101XR-01-136		2	gill	efishing	9.1	158.1
3_5_1	19101XR-01-137	19101XR-01-137NEB	2	blood	efishing	9.8	122
3_5_3	19101XR-01-138		2	muscle	efishing	9.5	225.2
3_5_5	19101XR-01-140		2	gill	efishing	9.2	238.1
3_6_3	19101XR-01-142		2	muscle	efishing	9.4	204.8
3_6_5	19101XR-01-144		2	gill	efishing	8.8	172.2
3_7_1	19101XR-01-145		2	blood	efishing	9.8	195.7
3_7_3	19101XR-01-146		2	muscle	efishing	9.2	329.3
3_7_5	19101XR-01-148		2	gill	efishing	9.2	270.4
3_8_1	19101XR-01-149		2	blood	efishing	9.8	139.5

Sample ID	QuantSeq	NEB ID	Group #	Tissue	Sampling Method	RIN	Concentra
3_8_3	19101XR-01-150		2	muscle	efishing	9.4	206.8
3_8_5	19101XR-01-152		2	gill	efishing	9.2	299.4
3_9_1	19101XR-01-153	19101XR-01-153NEB	2	blood	efishing	9.6	199.7
3_9_3	19101XR-01-154		2	muscle	efishing	9.5	161.6
5_1_1	19101XR-01-201		3	blood	efishing	9.1	20
5_1_3	19101XR-01-202		3	muscle	efishing	8.9	167.3
5_1_5	19101XR-01-204		3	gill	efishing	9.4	129.7
5_10_1	19101XR-01-237	19101XR-01-237NEB	3	blood	efishing	9.6	133.3
5_10_3	19101XR-01-238		3	muscle	efishing	9.4	642.9
5_10_5	19101XR-01-240		3	gill	efishing	9.4	147.7
5_2_1	19101XR-01-205		3	blood	efishing	9.7	66.6
5_2_3	19101XR-01-206		3	muscle	efishing	9	506.6
5_3_1	19101XR-01-209	19101XR-01-209NEB	3	blood	efishing	9.6	222.1
5_3_3	19101XR-01-210		3	muscle	efishing	9.4	275
5_3_5	19101XR-01-212		3	gill	efishing	8.9	935.2
5_4_1	19101XR-01-213		3	blood	efishing	9.7	71
5_4_3	19101XR-01-214		3	muscle	efishing	9.1	280
5_4_5	19101XR-01-216		3	gill	efishing	9.2	570.5
5_5_5	19101XR-01-220		3	gill	efishing	9.4	370.9
5_6_1	19101XR-01-221	19101XR-01-221NEB	3	blood	efishing	9.7	73.7
5_6_3	19101XR-01-222		3	muscle	efishing	9.3	277.7
5_6_5	19101XR-01-224		3	gill	efishing	9.2	233
5_7_1	19101XR-01-225		3	blood	efishing	9.4	112.2
5_7_3	19101XR-01-226		3	muscle	efishing	9.6	31.7
5_7_5	19101XR-01-228		3	gill	efishing	9.1	96.9
5_8_1	19101XR-01-229		3	blood	efishing	9.7	68.9
5_8_3	19101XR-01-230		3	muscle	efishing	9.6	156.1
5_8_5	19101XR-01-232		3	gill	efishing	9.5	68.7
5_9_1	19101XR-01-233	19101XR-01-233NEB	3	blood	efishing	9.7	212.3
5_9_3	19101XR-01-234		3	muscle	efishing	9.8	60.5
5_9_5	19101XR-01-236		3	gill	efishing	9	115.5

Table 2: Sample size used for each comparison . Sample size for the samples for which transcriptomic data were obtained divided for tissue type, treatment group, and library preparation is also indicated. Group number is as in Table 1: Group 1 – dip net with tissue harvested immediately after death; Group 2 – efishing with tissue harvested immediately after death; Group 3 – efishing with tissue harvested 5 min after death.

Comparisons			
Group1 vs Group2 QuantSeq	**Blood**	**Gills**	**Muscle**
Ntot=54	Ntot=17	Ntot=18	Ntot=19
N1=28	N1=10	N1=9	N1=9
N2=26	N2=7	N2=9	N2=10
Group2 vs Group3 QuantSeq	**Blood**	**Gills**	**Muscle**
Ntot=53	Ntot=16	Ntot=18	Ntot=19
N2=26	N2=7	N2=9	N2=10
N3=27	N3=9	N3=9	N3=9
NEB	**Group 1vs Group 2**	**Group 2vs Group 3**	
Ntot = 14	Ntot=10	Ntot=9	
	N1=5	N2=5	

Comparisons		
	N2=5	N3=4
QuantSeq vs NEB		
Ntot = 28		
NQuantSeq = 14		
NNEB=14		

Table 3: Comparisons for which changes in gene expression was tested .

	Sampling Method	**Time of Tissue harvesting**
Comparisons	Dip-netting vs electrofishing	Electrofishing and processed immediately vs 5 minutes after death
Library data Used	QuantSeq	QuantSeq
	NEB	NEB
	QuantSeq vs NEB	QuantSeq vs NEB

Figure Legends

Figure 1. Bar plot of transcript length versus number of non-expressed/non-detected genes for each RNA-Seq library technique. Data based on the 14 blood samples processed and sequenced using both library types. The plot only depicts genes that have been annotated with a known gene length.

Figure 2. 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.** QuantSeq dip netting versus electrofishing for all tissues combined, **B.** QuantSeq 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 QuantSeq comparisons for dip netting versus electrofishing only for blood samples, **F.** NEB versus QuantSeq comparisons for electrofishing with immediate sampling versus electrofishing with delayed sampling only for blood samples.

Figure 3. PCA plots showing PC1 and PC2 for samples that are differentially expressed among sampling techniques, tissue harvesting time, and library preparation methods. Treatment groups compared are indicated in different colored symbols next to each PCA plot.**A.** QuantSeq dip netting versus electrofishing for all tissues combined, **B.** QuantSeq 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.

Figure 4. PCA plots showing PC1 and PC2 for samples that are differentially expressed among sampling techniques, tissue harvesting time, and library preparation methods. Treatment groups compared are indicated in different colored symbols next to each PCA plot.**A.** NEB versus QuantSeq comparisons for dip netting versus electrofishing only for blood samples, **B.** NEB versus QuantSeq comparisons for electrofishing with immediate sampling versus electrofishing with delayed sampling only for blood samples.

Figure 5. Violin and box plots comparing gene expression versus gene length for NEB and QuantSeq library types. Each individual plot compares the number of genes with significantly different base mean expression for NEB versus QuantSeq, calculated as logbasemean NEB - logbasemean QuantSeq. Genes with equal expression fall on the zero line of the y-axis; genes with higher expression for the whole mRNA transcriptome versus QuantSeq have positive numeric values above 0, while genes with higher expression for QuantSeq vs whole mRNA transcriptome have negative numeric values below 0. The plot only depicts genes that have been annotated with a known gene length.

Supporting Information Legends:

Figure S1: 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. Group 1 = fish captured by dip netting, Grou[2 = fish captured by electrofishing processed immediately, Group 3 = fish captured by electrofishing processed 5 minutes after euthanasia.**A.** QuantSeq dip netting versus electrofishing only for blood samples, **B.** QuantSeq dip netting versus electrofishing only for gill samples, **C.** QuantSeq dip netting versus electrofishing only for muscle samples, **D.** QuantSeq electrofishing with immediate sampling versus electrofishing with delayed sampling only for blood samples, **E.** QuantSeq electrofishing with immediate sampling versus electrofishing with delayed sampling only for gill samples, **F.** QuantSeq electrofishing with immediate sampling versus electrofishing with delayed sampling only for muscle samples.

Figure S2: PCA plots showing PC1 and PC2 for samples that are differentially expressed among sampling techniques and tissue harvesting time for the different tissues. A. QuantSeq dip netting versus electrofishing only for blood samples, **B.** QuantSeq dip netting versus electrofishing only for gill samples, **C.**QuantSeq dip netting versus electrofishing only for muscle samples,**D.** QuantSeq electrofishing with immediate sampling versus electrofishing with delayed sampling only for blood samples, **E.**QuantSeq electrofishing with immediate sampling versus electrofishing with delayed sampling only for gill samples, **F.** QuantSeq electrofishing with immediate sampling versus electrofishing with delayed sampling only for muscle samples.