Biological Conservation 220 (2018) 1–11

Contents lists available at ScienceDirect

Biological Conservation

journal homepage: www.elsevier.com/locate/biocon

Concentrations of environmental DNA (eDNA) reflect spawning salmon abundance at fine spatial and temporal scales

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ARTICLE INFO

Keywords:
eDNA
Conservation genetics
Fish
Pacific salmon
Water sampling

ABSTRACT

Developing fast, cost-effective assessments of wild animal abundance is an important goal for many researchers, and environmental DNA (eDNA) holds much promise for this purpose. However, the quantitative relationship between species abundance and the amount of DNA present in the environment is likely to vary substantially among taxa and with ecological context. Here, we report a strong quantitative relationship between eDNA concentration and the abundance of spawning sockeye salmon in a small stream in Alaska, USA, where we took temporally- and spatially-replicated samples during the spawning period. This high-resolution dataset suggests that (1) eDNA concentrations vary significantly day-to-day, and likely within hours, in the context of the dynamic biological event of a salmon spawning season; (2) eDNA, as detected by species-specific quantitative PCR probes, seems to be conserved over short distances (tens of meters) in running water, but degrade quickly over larger scales (ca. 1.5 km); and (3) factors other than the mere presence of live, individual fish — such as location within the stream, live/dead ratio, and water temperature — can affect the eDNA-biomass correlation in space or time. A multivariate model incorporating both biotic and abiotic variables accounted for over 75% of the eDNA variance observed, suggesting that where a system is well-characterized, it may be possible to predict species' abundance from eDNA surveys, although we underscore that species- and system-specific variables are likely to limit the generality of any given quantitative model. Nevertheless, these findings provide an important step toward quantitative applications of eDNA in conservation and management.

1. Introduction

All organisms shed bits of DNA into their surrounding environments, leaving behind a genetic footprint comprised of skin, scales, waste, and other tissues, collectively referred to as environmental DNA or eDNA. In aquatic environments, eDNA remains suspended in the water, where it can be collected, extracted, and sequenced/amplified, to reveal the habitat's species composition (Díaz-Ferguson and Moyer, 2014; Rees et al., 2014; Thomsen and Willerslev, 2015). Over the past decade, the use of eDNA technologies in the conservation and management of animal populations has evolved from a novelty to a valuable tool for scientists and resource managers (Jones, 2013; Kelly et al., 2014b). In comparison with traditional means of sampling taxa, eDNA technologies may be more efficient with regard to both time and money, and can overcome the practical and regulatory challenges associated with capturing rare or endangered species (Evans et al., 2017; Shaw et al., 2016). Indeed, in some cases, eDNA can generate substantially more information on species in less time than traditional survey methods (Ji et al., 2013; Valentini et al., 2015), such as alpha and beta diversity (e.g. Kelly et al., 2016). Furthermore, the rapid reduction in costs associated with genetic research over the past decades makes genetic approaches increasingly cost-effective.

At present, eDNA sampling is being applied extensively for the detection of invasive (Fujiwara et al., 2016; Jerde et al., 2011; Takahara et al., 2013) and endangered (Laramie et al., 2015a; Thomsen et al., 2012; Wilcox et al., 2013) species, and for the estimation of biodiversity across terrestrial, aquatic, and marine environments (Kelly et al., 2014a; Lodge et al., 2012; Port et al., 2016). These applications of eDNA all rely on its ability to detect the presence or absence of one or more species in a given environment. As such, the usefulness of eDNA methods remain limited because effective conservation of threatened species and management of exploited populations often requires estimates of abundance in addition to knowledge of presence and distribution. Consequently, development of eDNA sampling and analysis

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https://doi.org/10.1016/j.biocon.2018.01.030
Received 30 July 2017; Received in revised form 12 January 2018; Accepted 24 January 2018

protocols that allow for estimation of relative or absolute abundance will greatly expand the applicability of the technology for conservation and management, and remains an important frontier in eDNA research (Kelly, 2016).

Recent examples of quantitative eDNA applications highlight both the promise and challenge of abundance estimation using these technologies. Kelly et al. (2014a) and Evans et al. (2016) both found positive correlations between eDNA concentration and biomass of multiple species in mesocosm settings using a multispecies metabarcoding approach. However, in both cases, despite known abundance and biomass, the relationships were generally weak and varied markedly between taxa. Targeted analyses using species-specific probes and qPCR avoid some of the potential biases of multi-species methods, and recent field studies have reported significant relationships between abundance or biomass and eDNA quantity using this approach. For example, Lacoursière-Roussel et al. (2016) found a weak, but positive relationship between catch per unit effort of lake trout (*Salvelinus namaycush*) and concentrations of the species' eDNA as detected using qPCR in several large lakes in Canada. Doi et al. (2017) detected a significant, positive relationship between snorkel-survey counts of the stream-dwelling fish *Plecoglossus altivelis* and eDNA concentrations in the Saba River, Japan. The direction of the relationship remained constant across seasons, but the slope increased markedly in the fall, highlighting the potential for temporal variability in the processes of eDNA production, transport or degradation. Buxton et al. (2017) also found a positive relationship between great crested newt (*Triturus cristatus*) abundance and eDNA concentrations in a series of ponds. However, they detected seasonal dynamics in eDNA concentration independent of abundance (likely a result of breeding activity). The results of these and other recent studies (e.g., Baldigo et al., 2017; Pilliod et al., 2013; Takahara et al., 2012) suggest that estimating abundance of aquatic organisms with eDNA approaches may indeed be possible.

Quantitative applications of eDNA would be particularly valuable in conservation of salmon and other anadromous fishes. Productive populations of these species are often exploited at high rates and managed using data-intensive approaches. Other populations are severely depleted and of significant conservation concern with many listed as endangered or threatened (Quinn, 2005). In both cases, estimates of abundance at various stages of the life cycle provide valuable data on survival, movement, habitat quality, and population productivity that directly inform management and conservation efforts. Traditional methods of salmon enumeration include weirs, counting towers, mark-recapture studies, float/walking/aerial surveys and hydroacoustics (Enzenhofer et al., 1998; Holt and Cox, 2008). These and other counting methods can be expensive and are often labor-intensive, limiting the spatial and temporal extent of monitoring by management agencies with finite budgets. The extent or resolution of monitoring efforts would be greatly increased if robust abundance estimates could be derived from eDNA samples, benefiting fish populations and the ecosystems, individuals, and communities that rely upon them. However, despite recent progress in quantitative applications of eDNA sampling, many uncertainties remain regarding the generation, transport and degradation of DNA in the environment (Andruszkiewicz et al., 2017; Barnes and Turner, 2015; Civade et al., 2016; Deiner and Altermatt, 2014; Jerde et al., 2016; Sassoubre et al., 2016; Shogren et al., 2016), and thus the spatial and temporal resolution of eDNA-based abundance estimates. For example, shedding rates may vary seasonally (Buxton et al., 2017) or with age (Maruyama et al., 2014) and degradation rates may be correlated with environmental factors such as temperature and microbial activity (Lance et al., 2017). Many of these issues are particularly acute for spawning salmonids occupying dynamic stream environments and undergoing rapid physiological, behavioral and morphological changes.

While the ultimate goal of quantitative eDNA methodologies is to estimate animal abundance from DNA concentration, the studies described above demonstrate such correlations to be both variable and uncertain. Reversing this model, and instead examining biological and environmental factors that influence the amount of DNA detectable in the environment is a key step in improving the predictive power of eDNA-based quantification. In this study, we sought to address some of these key uncertainties by exploring the quantitative relationship between eDNA and the abundance of sockeye salmon (*Oncorhynchus nerka*) in Hansen Creek, a small tributary of Lake Aleknagik near Bristol Bay, Alaska, USA. Counts of live and dead fish in the creek have been conducted daily during the spawning season – typically mid-July through August – for over 20 years. As such, the timing and spatial extent of the spawning run is well understood, and there is an established methodology for assessing the salmon population. Moreover, sockeye salmon are semelparous, so all adults die at the end of the spawning season rather than remaining in the stream, as would be the case for most fishes. These features, combined with physical characteristics making it amenable to visual surveys of salmon, make Hansen Creek attractive for exploring the relationship between eDNA and fish abundance in a natural setting. In order to examine the relationship between sockeye salmon abundance and the amount of sockeye salmon eDNA in the water we tested three hypotheses:

1. eDNA does not substantially degrade or settle, or become entrained in sediment along the length of Hansen Creek, and therefore the furthest-downstream sampling site will consistently record the highest concentrations of eDNA.
2. eDNA from tributaries will be additive, so that eDNA sampled on separate tributaries upstream of a confluence should approximately equal eDNA sampled just downstream of the confluence.
3. eDNA concentration is linearly related to the total number of fish in Hansen Creek and inversely related to temperature.

2. Methods

2.1. Field methods

Hansen Creek is a small stream located in the Wood River watershed of southwest Alaska. It is well suited for exploring eDNA dynamics for several reasons. The spring-fed creek is both small (~2 km long and averaging 4 m wide and 10 cm deep) and simple, with only one significant tributary entering from a spring-fed pond (hereafter “side pond”) ~0.5 km downstream of the headwater pool created by an old beaver dam (Fig. 1; Quinn and Buck, 2001). Adult sockeye salmon occupy the stream only during the spawning period, beginning in mid-July and concluding before the end of August. Juvenile sockeye salmon rear in lakes and typically leave their natal streams in the spring, and so by mid-July few if any remain in Hansen Creek when adults return to spawn. Over several decades of spawning season surveys no other Pacific salmon species have been documented in the creek, and resident fishes (e.g., cottids, juvenile Dolly Varden and Arctic char) are small-bodied and present only in small numbers. Because it is spring-fed and low-gradient, discharge in the stream remains relatively constant during the spawning season (mid-July to mid-August), even following rain events (Tillotson and Quinn, 2017). These physical and biological characteristics also make it highly amenable to visual surveys of spawning salmon (Quinn et al., 2014).

As part of an independent, long-term research program, daily visual counts of live and dead sockeye salmon occur in Hansen Creek during the spawning season using well established protocols (Quinn et al., 2014; Quinn and Buck, 2001; Tillotson and Quinn, 2017) which we followed. For our purposes, the relevant information provided by these surveys included daily estimates of live, naturally dead (senescent fish that successfully spawned) and killed sockeye (largely from bear predation) present in five different reaches of Hansen Creek (lower (~1100 m), middle (~580 m), side pond (~890 m^2), upper (~440 m), beaver pond (~4200 m^2); Fig. 1). Also important for this study, in accordance with the long-running study site's standard protocol (e.g.,

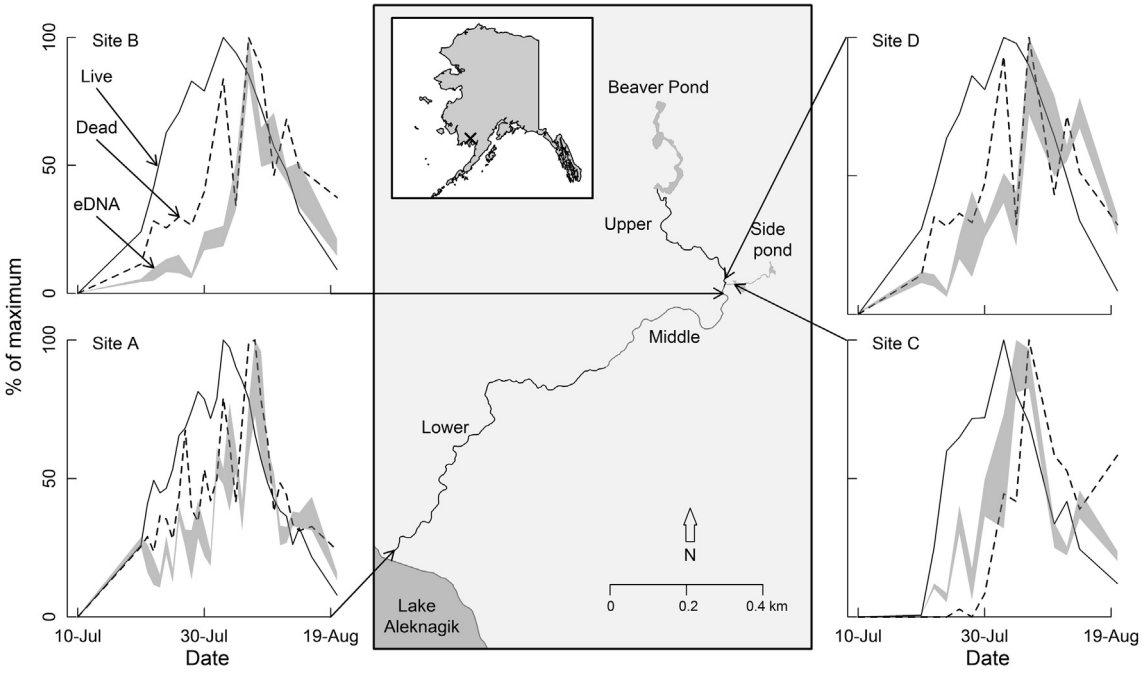

Fig. 1. Map of Hansen Creek, Alaska (indicated by an X on the state map) showing locations of eDNA sampling sites and visual count survey reaches. Peripheral panels show temporal trends in eDNA rate (shaded grey area; 95% confidence interval) and salmon abundance (live: solid lines, dead: dashed lines) for each sampling site. y-Axes are scaled relative to their maximum observed values to facilitate visual comparison between salmon counts and eDNA rates.

Quinn et al., 2014), all dead sockeye salmon were removed from the creek during surveys to prevent double counting and thrown ~5 m or more from the channel. eDNA sampling was conducted during the daily visual surveys. In order to reduce the possibility of contamination, samples were always collected prior to any researchers proceeding upstream of an eDNA sampling site, and thus collected while recently killed and naturally dead salmon (i.e., those since the last survey) were in and near the stream.

We sampled eDNA at four sites in Hansen creek: at the downstream edge of the lower survey reach below all spawning in the stream (site A, representing the entire stream), ~10 m downstream of the confluence with the side pond tributary (site B, representing the side pond, upper section, and beaver pond), ~5 m upstream from the confluence on the tributary (site C, representing the side pond), and the main stem (site D, representing the upper section and beaver pond). Triplicate 1-L stream water samples were collected at each site and filtered using a 0.45 µm cellulose nitrate filter with suction provided by a battery-powered drill and peristaltic pump. Pumping continued until the desired volume was achieved, or until flow ceased due to clogging (Laramie et al., 2015b). Filtered water was measured to the nearest 5 ml using a 1-L graduated cylinder, which allowed a consistent metric of eDNA concentration to be calculated even when filtered volume varied between samples. Results from Eichmiller et al. (2016) suggest that this is an appropriate correction to make as fish eDNA concentrations estimated in an experimental setting were statistically identical across a four-fold change in the volume of water filtered. Filters were then preserved in ethanol. At the end of each sampling day a negative control was collected by following the field sampling procedure using 1-L of distilled water. All field equipment that came into contact with the sample was single-use and disposable.

At each eDNA sampling site environmental parameters were collected prior to water filtering. A YSI Pro20 dissolved oxygen meter was used to measure temperature and dissolved oxygen near the thalweg. A pressure transducer was installed in the lower reach of the stream throughout the sampling season to record water depth, which was then corrected for variation in atmospheric pressure. Based on a rating curve previously established for Hansen Creek, hourly water depths were converted to discharge (liters/s) and averaged across each day.

2.2. Laboratory analysis

2.2.1. eDNA sample preparation

We employed stringent quality control and quality assurance practices to prevent sample contamination, which included separating work flow of eDNA sample preparation into designated work rooms and following the recommendations for "clean practices in the laboratory" in Goldberg et al. (2016). DNA collected on filters was extracted using Qiagen DNeasy Blood & Tissue kits with the following modifications: 360 µl ATL buffer and 40 µl Proteinase K were used for cell lysis and the volume of AL buffer and 100% ethanol was adjusted to 400 µl post-lysis. DNA was eluted in 120 µl AE buffer and stored at −20 °C until qPCR analysis.

Before qPCR sample analysis, all DNA extracts were tested for the presence of PCR inhibitors. An internal positive control (IPC) assay was performed in triplicate on each DNA extract using TaqMan Exogenous Internal Positive Control Reagents (EXO-IPC; Applied Biosystems). Each IPC assay consisted of 5 µl TaqMan Gene Expression MasterMix, 3 µl eDNA template, 1 µl 10× EXO-IPC mix and 0.22 µl EXO-IPC DNA per 10 µl total volume reaction. All qPCR analysis for this study including IPC analysis was performed on a ViiA7 Real-Time PCR System (Applied Biosystems). Cycling parameters consisted of initial steps of 2 min at 50 °C then 10 min at 95 °C, followed by 40 cycles of denaturing at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. We considered samples to have PCR inhibition when the mean IPC cycle threshold (C_t) value for a sample was greater than two C_t higher than the mean C_t value for the no-template control sample (Hartman et al., 2005). Samples that were identified to have reduced amplification were processed for the removal of PCR inhibitors using the OneStep PCR Inhibitor Removal Kit (Zymo Research). After secondary cleanup, DNA extracts were tested with the IPC and then used for subsequent qPCR analysis.

2.2.2. qPCR analysis

Environmental sockeye DNA was quantified using a protocol developed at the USGS Western Fisheries Research Center (Seattle, WA) using a TaqMan minor groove binding (MGB) assay (SECO3_861-930).

The SECO3_861-930 primers (forward: 5′-TCTGCCCTTCTCCTTACGATTTT-3′ and reverse: 5′-GTTCGACCTAGAAATCGCCCTT-3′) and a FAM-labeled MGB non-fluorescent quencher probe (6FAM-5′-CCATCCTGTTCCTCCT-3′-MGBNFQ) amplifies a 70 base pair region of the mtDNA Cytochrome c oxidase subunit III gene, and was designed using Primer Express 3.0 (Applied Biosystems; development and validation are reported in Appendix A). SECO3_861–930 qPCR assays consisted of 1 × TaqMan Gene Expression MasterMix (Life Technologies), 1 × custom TaqMan primer and probe mix (450 nM each forward and reverse primer and 125 nM probe) and 3 µl eDNA extract in 10 µl total volume reactions. Negative controls (store-purchased water filtered in-field, DNA extraction negative controls and no-template controls) were included in the qPCR runs to detect contamination. qPCR on each eDNA extract, control and standard was performed in triplicate, resulting in 9 total qPCRs per sampling event (3 water samples/sample site × 3 qPCR wells/water sample). We used Ultramer DNA Oligonucleotides (Integrated DNA Technologies), which is a synthetic gene of our target gene fragment (the amplicon, containing primer and probe sites), to create a copy number standard curve. A five-point curve consisting of a 1:5 serial dilution of 10,000, 2000, 400, 80 and 16 copies per reaction was used for sample copy number quantification. The mean assay efficiency was 86.08%, $R^2 > 0.99$ and the Limit of Detection was calculated to be 9.38 copies at C_t 35.884. Mean (± SD) mtDNA copy number was estimated across the three replicate water samples for each sampling event.

Variation between the three copy number estimates for each field sample was typically low, but when the coefficient of variation (CV) between replicates exceeded 0.25, three additional qPCR replicates were conducted. If the CV decreased in the second analysis the new values were used for further analysis (27 of 30 re-run triplicate samples of a total 117 run experiment-wide), otherwise the original values were used. The numbers of DNA copies estimated by qPCR analysis were standardized by first dividing the quantity value by the total volume of water filtered which was recorded in the field giving DNA concentration (DNA copies/l). To account for spatial and temporal variability in stream discharge (and thus dilution of DNA) we calculated the number of DNA copies flowing past the sampling site per second; hereafter referred to as DNA rate. Rates were calculated as the product of DNA concentration (DNA copies/l), daily discharge (l/s) and the average proportion of total stream flow in each tributary: 100% downstream of side pond confluence (sites A, B), 12% in side pond tributary (site C) and 88% in mainstem upstream of confluence (site D). DNA rates are reported as thousands of DNA copies per second (copies · 10^3/s).

2.3. Data analysis

2.3.1. Spatial resolution of eDNA in flowing water

Studies of eDNA in lotic environments have found a wide range of effective transport distances (e.g., ~12 km in Deiner and Altermatt (2014); 50% degradation within 100 m in Wilcox et al. (2016)). Despite this large uncertainty in the rate of degradation, because Hansen Creek is relatively cool and water residence time is a matter of hours, we hypothesized that DNA degradation and settling/entrainment would be minimal. If eDNA is indeed conserved over the spatial scale of our study system, samples collected at the mouth of the creek would reflect total salmon abundance in the entire creek. To test this hypothesis, we compared eDNA rates between site A – the furthest-downstream site – and site B which is located ca. 1.5 km upstream. If eDNA degrades minimally in Hansen Creek then the eDNA rate at site A should be equal to or greater than that at site B; most likely greater because many additional fish are typically present downstream of site B. Thus, we first plotted eDNA rates at each site over time to allow for visual comparison. To evaluate the statistical significance of any visually observed differences we then conducted a one-tailed paired Student's *t*-test with the alternative hypothesis that on each sampling day, site A DNA copies · 10^3/s > site B DNA copies · 10^3/s.

2.3.2. Additivity of eDNA from independent sources

We compared the sum of eDNA rates from sites C and D (which are located on separate tributaries of the creek and therefore downstream of independent numbers of salmon) with the eDNA rate at site B, located just downstream of the confluence of these two DNA sources. We hypothesized that the sum of eDNA rates from C and D would be approximately equal to site B given the small distances between sites and similar water quality in both tributaries. We followed the same procedure as above, first visually inspecting plots of eDNA rate over time and then conducting a paired Student's *t*-test; in this case the two-tailed variety which tests for differences in either direction (i.e., site B DNA copies · 10^3/s ≠ site C + D DNA copies · 10^3/s).

2.3.3. eDNA in relation to abundance and environmental factors

To evaluate the relative influence of environmental and biological factors on eDNA rate in Hansen Creek, we fit linear models using generalized least squares (GLS). Following the backward model selection procedure described in Zuur et al. (2009), we first evaluated our data for violations of homogeneity and found unequal variances between sites and increasing variance with increasing salmon abundance. As such, all models used in later comparisons included a residual variance structure that accounted for this heterogeneity. We then worked backward from a model containing all possible fixed and interaction terms and compared AIC values to identify the model that best balanced explanatory power with parameterization. AIC was calculated as $2k - 2\ln(L)$ where k is the number of model parameters and $\ln(L)$ is the log-maximum-likelihood estimate for the model. The relative strength of models was further compared using Akaike weights calculated as $\exp(-0.5\ (AIC_{best} - AIC_i))$. Potential explanatory variables included counts of live, dead and killed salmon, temperature, dissolved oxygen (DO) and site (a factor). To evaluate the relative DNA contribution of live and dead fish, we also considered counts at different levels of aggregation: dead includes both naturally dead and killed fish, total includes all fish live and dead. Table 2 shows all candidate models.

3. Results

3.1. Season summary

We began eDNA sampling on 10 July 2016 to measure background DNA levels, a date well before the long-term average arrival of the first adult sockeye to Hansen Creek (Carlson and Quinn, 2007). Based on historical observations and a lack of any signs of live or dead adult sockeye salmon in the creek, we are very confident in our zero-count of adults on this date. Nevertheless, sockeye DNA was detected in all sites and in all replicates, though at levels (0.99–5.62 copies · 10^3/s) at least an order of magnitude lower than those measured after the arrival of adults which occurred between 10 July and 20 July. There are several possible explanations for these positive detections including the presence of late-migrating fry in the creek, residual egg casings in the gravel, resuspension of eDNA in sediments (Barnes et al., 2014) or field equipment contamination. Beginning on 21 July eDNA sampling and visual count surveys were conducted daily through 14 August, and then again on 16 August and 20 August. Site A was sampled on all days for eDNA and sites B, C and D were sampled approximately every other day.

The temporal trends in eDNA rate and fish abundance were both unimodal over the course of the spawning season, generally increasing until the first week of August and then decreasing through the end of the study (Fig. 1). Total daily counts of live and dead adult sockeye salmon in the entire creek ranged from 0 to 2286, peaking on 2 August. eDNA rate peaked at all sites between 4 and 7 August, two to five days following peak salmon abundance. We were unable to sample after 20 August due to logistical constraints even though 155 live salmon remained in the stream at the end of the study. eDNA rate had declined markedly from its peak by this date, but we were unable to make a

Table 1
Summary of physical characteristics and eDNA results by site.

	Days	Volume (m^3)	Density (salmon/m^3)		eDNA concentration (copies/ml)			% flow	eDNA rate (copies $\cdot 10^3$/s)		
			Median	Max.	Min.	Median	Max.		Min.	Median	Max.
A	29	2429	0.46	1.02	0.02	6.38	22.54	100	0.99	326	1104
B	15	1986	0.47	0.77	0.11	8.35	32.22	100	5.15	420	2121
C	14	167	1.16	1.93	0.14	20.53	82.05	12	0.91	123	510
D	14	1819	0.42	0.66	0.13	8.22	23.54	88	5.62	362	1170

Days – number of days sampled for eDNA, Volume – estimated volume of water upstream of sampling site, Density – density of salmon per cubic meter upstream of sampling site; note that minimum is 0 for all sites, % flow – the relative portion of streamflow passing each sampling site; the main channel below the confluence with the side pond tributary is considered 100%.

comparison between pre- and post-season samples with zero-counts. Table 1 provides summaries of count and eDNA data for each site.

3.2. Spatial resolution of eDNA in flowing water

eDNA rate at site A (downstream, near the mouth of the creek) ranged from 0.993 to 1104 copies $\cdot 10^3$/s across 86 samples, while at site B (upstream, at the confluence of tributaries) the range was 5.148 to 2121 copies $\cdot 10^3$/s across 45 samples. Of the 15 days that both sites were sampled, DNA rate was greater at site A on six days and greater at site B on nine days. Our hypothesis that in the absence of significant DNA degradation or entrainment, DNA rate at site A should always be higher than at site B was rejected using a one-sided, paired Student's *t*-test ($t = -4.07, p = 0.99$). However, a two-sided *t*-test comparing daily means indicated that DNA rates were significantly different between sites on each day, with site B on average 256.6 (95% CI: $\mp$ 127.2) copies $\cdot 10^3$/s greater than site A ($t = -4.07$, $p < 0.001$). Indeed, especially during the second half of the spawning season, DNA rate was much higher at site B despite the fact that many salmon were present in the stream between sites B and A (Fig. 2). This suggests that perhaps more than half of the total DNA passing site B was lost to degradation or settling/entrainment in the lower ~1.5 km of stream. Fig. 2 (right panel) shows the temporal trends in DNA rate at sites A and B and Fig. 3 (right panel) shows the linear relationship between DNA rates at the two sites.

3.3. Additivity of eDNA from independent sources

We tested the additive relationship of two separate sources of eDNA by comparing eDNA rate at site B with the sum of the rates at sites C and D. As shown in Fig. 1, sites C and D are both < 30 m upstream of site B, but on separate branches of the stream and therefore measure independent sources of sockeye salmon eDNA. The sum of eDNA rates at sites C and D ranged from 6.849 to 1659 copies $\cdot 10^3$/s, overlapping substantially with the range for site B (5.148 to 2121 copies $\cdot 10^3$/s). A point-by-point comparison using a two-sided Student's *t*-test indicated no difference in daily means ($t = 0.33$, $p = 0.75$). Fig. 2 (left panel) shows the temporal trends in DNA rate for site B and the sum of sites C and D; Fig. 3 (left panel) shows the linear relationship between DNA rates at the two sites. Thus, the concentration of sockeye eDNA at the confluence of the two tributaries (site B) reflected the sum of the concentrations in each tributary (C and D) when measured at a fine spatial scale (ca. 30 m apart).

3.4. eDNA in relation to abundance and environmental factors

Our AIC model-selection procedure indicated strong support for a model that included live, natural-dead, and killed fish separately, along with site-specific intercepts and site-specific slopes for live and natural-dead. This model was strongly preferred over models that aggregated killed and naturally dead fish or treated all three states of fish as the same, indicating that fish in each state released eDNA at different rates (Table 2). This model was also preferred over models that did not include site, suggesting that eDNA dynamics are dependent on local hydrological characteristics and/or the location of eDNA sampling relative to concentrations of fish. Overall, the selected model explained over 75% of the variance in eDNA rate (Fig. 4). In the AIC selected model, the strongest predictor of eDNA rate was naturally dead fish for which each additional fish increased eDNA rate by 3.40 $\pm$ 0.33 copies $\cdot 10^3$/s at site A, 11.16 $\pm$ 1.14 at site B, 7.63 $\pm$ 1.44 at site C and 10.23 $\pm$ 1.14 at site D. For all sites, each killed fish increased eDNA rate by 1.18 $\pm$ 0.31 copies $\cdot 10^3$/s, and for a 1 °C increase in temperature, eDNA rate decreased by 14.16 $\pm$ 5.07 copies $\cdot 10^3$/s. Finally, the influence of live fish varied by site, with each fish increasing eDNA by 0.69 $\pm$ 0.15 copies $\cdot 10^3$/s at site C and having no effect on eDNA at site A (0.06 $\pm$ 0.05), site B (0.07 $\pm$ 0.09) or site D (-0.02 ± 0.07).

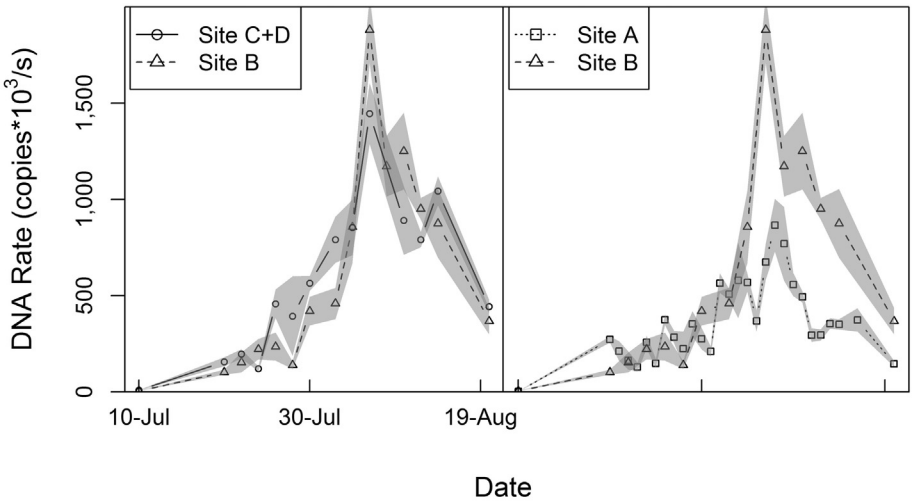

Fig. 2. Comparison of temporal trends in DNA rate between site B and the sum of sites C and D (left panel) and between sites A and C (right panel). Shaded areas show 95% confidence intervals.

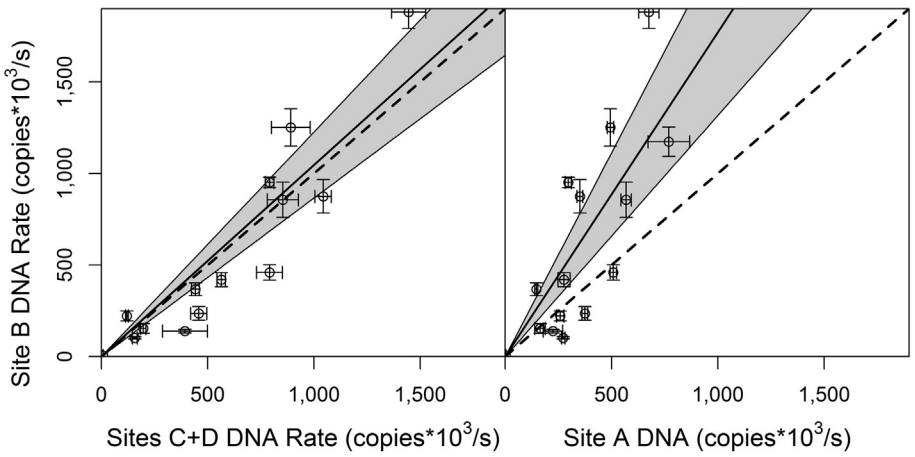

Fig. 3. Correlations between DNA rate at site B and 1) sites C + D (left panel), 2) site A (right panel). Dashed line shows the hypothesized 1–1 line for each relationship. Solid line and shaded areas show best fit slope only models with 95% confidence intervals. Points are data and error bars show standard errors.

Based on the Akaike weights, two other models received minor support though each contained all the variables found in the best model and did not appreciably change the coefficients given above (Table 2).

4. Discussion

Using high-resolution sampling (in both space and time) in a well-characterized field setting, we addressed important unknowns surrounding the quantitative relationship between species abundance and eDNA concentration, and the spatial and temporal resolution of the technique. Supporting the idea that eDNA sampling may be suitable for enumeration of salmon in streams, we found a strong correlation between eDNA and abundance in Hansen Creek. More generally, we found that (1) eDNA concentrations varied significantly day-to-day, and likely within hours, in the context of a dynamic biological event such as a salmon spawning season; (2) eDNA, as detected by species-specific quantitative PCR probes, seemed to be conserved over short distances (tens of meters) in running water, but degrade quickly over larger scales (ca. 1.5 km); and (3) factors other than the mere presence of live, individual fish – such as spawning behavior, state (i.e. live, killed, naturally dead), and water temperature – can alter the eDNA-abundance correlation in space or time. Taken together, these results strengthen the view that eDNA monitoring from water samples provides a here-and-now look at local organisms, including information on their abundance, but that locally relevant habitat features or behaviors are likely to influence quantitative eDNA assessments of salmon and other animal species.

4.1. Temporal eDNA dynamics

DNA detected in an environmental sample – whether through amplicon sequencing or, as here, qPCR – is the net result of eDNA production minus a combination of degradation and transport. The amount of DNA an organism puts into the surrounding environment is due to sloughing, excretion, injury, or post-mortem disintegration. In the case of breeding fish such as adult salmon, the release of gametes may also contribute (Lance et al., 2017). Degradation is likely to be a function of biotic (microbial digestion) and abiotic (temperature, UV exposure) factors (Barnes and Turner, 2015), and transport in an aquatic or marine environments is likely to be via prevailing flow as perceived at the spatial scale of individual cells.

Given the relatively small size, swift flow and short residence time of water in Hansen Creek, we expected that samples collected approximately 24 h apart would be essentially independent and contain only eDNA from salmon present on the day of sampling. If true, then a plot of salmon eDNA over time should at least roughly match the temporal pattern of abundance. If this were not the case and eDNA persisted in the system over a longer timeframe, then a plot eDNA over time would be broader and lag behind the abundance curve. We observed significant day-to-day variation in eDNA concentration within each site, consistent with the idea of rapid production and degradation or transport (rather than with an alternative accumulating model). Additionally, the temporal plots of eDNA (Fig. 1) have stronger peaks than the abundance curves, further suggesting that sockeye salmon eDNA did not accumulate over time in Hansen Creek (factors that may explain the different shapes of the eDNA and abundance curves are discussed further in Section 4.3). Together, these results suggest that changes in abundance are reflected at quite fine time scales by eDNA concentrations. From the perspective of applying eDNA for abundance estimation, this is likely to beneficial as it implies that eDNA can provide a snapshot of current or very recent conditions.

4.2. Spatial eDNA dynamics

Because genetic material is likely to move quickly through space in lotic systems, the spatial resolution of eDNA sampling is also an

Table 2
Candidate models with AIC values and AIC weights.

Model	AIC	ΔAIC	Akaike weight	R^2
Live + Nat. Dead + Kill. + Site * Live + Site * Nat. dead + Temp.[a]	5711	0	0.92	0.76
Live + Nat. Dead + Kill. + Site * Live + Site * Nat. Dead + Site * Kill + Temp.	5716	5	0.06	0.76
Live + Nat. Dead + Kill. + Site * Live + Site * Nat. Dead + Temp. + DO	5718	7	0.02	0.76
Live + Nat. Dead + Kill. + Site * Nat. Dead + Temp.	5724	13	0	0.75
Kill. + Nat. Dead + Site * Nat. Dead + Temp.	5726	17	0	0.75
Live + Dead + Live * Site + Dead * Site + Temp.	5800	76	0	0.64
Live + Nat. Dead + Kill. + Site + Temp.	5820	112	0	0.59
Live + Nat. Dead + Kill. + Temp.[a]	5872	161	0	0.46
Total + Total*Site + Temp.	5897	185	0	0.38

[a] Comparison of these two models highlights the importance of site-specific effects; removal of interaction terms drastically reduces the predictive power of the top model.

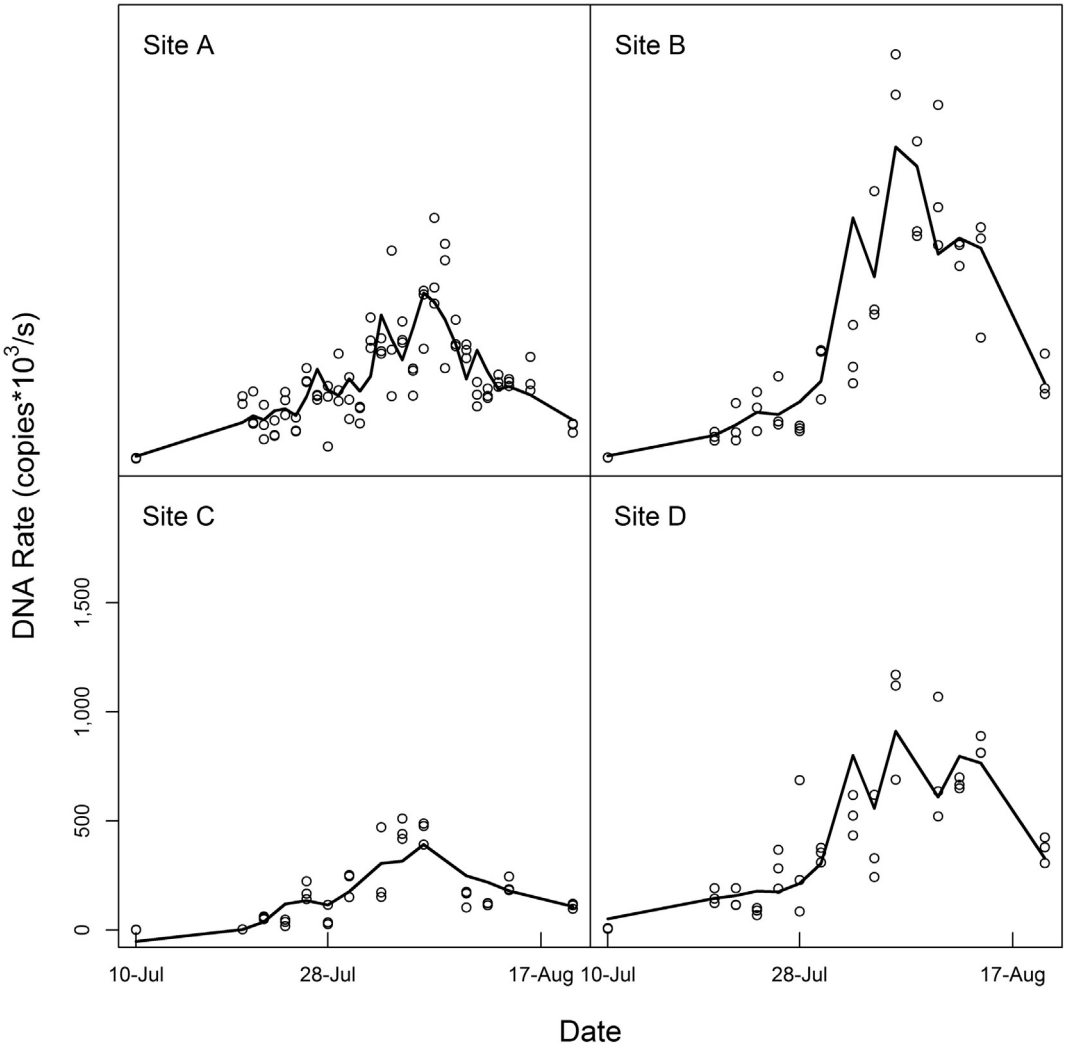

Fig. 4. AIC selected model predictions by site. Points are observed DNA rates and lines are predictions from strongly favored best-fit model; model details shown in Table 2 and described in Section 3.4.

important component of estimating abundance. Although rivers can carry genetic material downstream (Deiner and Altermatt, 2014), many studies have found that riverine eDNA generally reflects organisms present nearby (Civade et al., 2016; Spear et al., 2015; Wilcox et al., 2016). Our comparison of eDNA at upstream and downstream sites supports the prediction that eDNA does not accumulate downriver (also see Jerde et al., 2016; Pilliod et al., 2014) as one would expect if the sum of production and transport exceeded degradation and storage (Site A, the downstream most site did not consistently have the highest eDNA rates). On the contrary, our results affirmed the idea that eDNA degrades rapidly in space and time (consistent with Jerde et al., 2016, Sassoubre et al., 2016, Andruszkiewicz et al., 2017) or at least is removed from detection (perhaps through settlement or stochastic binding; see Shogren et al., 2016). eDNA from nearshore marine environments appears to behave similarly (O'Donnell et al., 2017; Port et al., 2016).

The evidence therefore points to a large degradation or settlement term and an accordingly small effective transport term (transport of degraded material is ineffective) in a mass-balance view of eDNA dynamics. Under this hypothesis, nearly all recovered eDNA is generated in the immediate vicinity because it is degraded or settles nearly as quickly as an organism produces it, making detections increasingly unlikely as time or distance-from-source increases. Jane et al. (2015) and Wilcox et al. (2016) modeled and observed eDNA transport and degradation in salmonids in streams analogous to Hansen Creek, finding a) eDNA copy number declined exponentially with distance from source over tens to hundreds of meters, and b) faster-moving creeks are likely to carry eDNA further. Given that Hansen Creek's discharge rate is ~100–500 L/s (Tillotson and Quinn, 2017), the results of Wilcox et al. (2016) suggest that the transport distance of detectable eDNA in Hansen Creek should be > 200 m. Although our study lacked the fine-scale resolution that would allow us to test this hypothesis precisely, our results are broadly consistent with an effective transport distance (i.e., detection by qPCR) of hundreds of meters, in accordance with Jane et al. (2015) and Wilcox et al. (2016). We found further support for this model of the spatial dynamics of eDNA by comparing samples from stream tributaries (sites C and D) to samples from their confluence (site B) and at the mouth of the creek (site A). Salmon eDNA concentrations at the confluence (B) contained the additive signal of the two tributaries (C and D, sampled at sites ca. 30 m apart). But as noted above, samples collected near the mouth (A) contained significantly less sockeye salmon eDNA than at the confluence (B), ca. 1.5 km upstream.

Our findings regarding both the spatial and temporal resolution of eDNA sampling support the notion that, at least in small streams, genetic material present in the environment originates from organisms that are relatively nearby, or have been in the very recent past. While these general observations are instructive, robust methods for estimating abundance from eDNA samples will necessitate analytical approaches that explicitly account for DNA transport and degradation rates. These analyses need not be overly complex; indeed, the relatively

simple mechanistic model used by Wilcox et al. (2016) in a study of eDNA detection probabilities may be suitable in many cases. Regardless of the analytical approach, careful study design with spatially standardized sampling locations should also improve the reliability of eDNA-based abundance estimates.

4.3. Biological and environmental influences on eDNA concentrations

Examination of the temporal eDNA and abundance trends in Fig. 1 shows that, although eDNA concentration followed the general pattern of fish abundance, there was some temporal disconnect, particularly from the live counts. Comparison of statistical models relating eDNA to fish counts and environmental covariates helped to clarify the causes of this apparent disconnect. In general, the modeling results confirmed the visually apparent pattern: eDNA concentration tracked the number of dead fish more closely than that of live fish. Modeling results indicated that eDNA concentrations in Hansen Creek were primarily driven by fish abundance and state (e.g. live vs. dead) with a minor effect of temperature (presumably, eDNA breaks down more rapidly at higher temperatures; Lance et al., 2017; Strickler et al., 2015), and with some variation in these relationships among sampling sites. This multivariate model outperformed (among many others) the simpler model formalizing our a priori expectations, in which variance in the total number of fish, independent of state would account for most of the variance in eDNA concentration. We were able to explain most of the variance in eDNA with a few easily-observed variables, suggesting a tight mechanistic connection between the salmon run and the genetic traces the fish leave behind. However, the complexity of the best-fit model also suggests that, at least in Hansen Creek, inferring live-salmon counts would not as straightforward as measuring salmon eDNA concentration.

One challenge demonstrated in our modeling results is the influence of local hydrological and physical habitat characteristics on the relationship between eDNA and species abundance. The significance of interactions terms between fish counts (i.e., live, natural dead) and site in the selected model indicates that the relationship between salmon abundance and eDNA available for sampling varies by location (removal of site specific factors from the AIC-selected model decreased the R^2 from 0.76 to 0.46; Table 2). This is likely in part because – despite the rapid degradation of eDNA from upriver – there was still some influence from upstream eDNA at downstream sites. In addition, sites differed considerably in hydrology; upstream sampling sites were located much closer to large ponds where eDNA may behave quite differently than in free-flowing reaches. For example, Site D was located downstream from a pond that often contained over 1000 fish, whereas site A was at the terminus of over 1.5 km of free-flowing stream holding a lower density of sockeye salmon. These kinds of physical and biological details are perhaps sensible in understanding the process leading from fish to genetic signal for sockeye salmon in Hansen Creek. However, the dependence of the model on these details suggests a larger point about eDNA quantification in the natural world: it is difficult to generalize across species and across habitats with current methodologies.

A particular challenge for predicting Pacific Salmon abundance is likely to be the differential contribution of eDNA from live and dead fish, which is apparently substantial. Indeed, based on all of the most parsimonious models (Table 2) dead fish apparently released substantially more eDNA than live fish. This result was somewhat unexpected, and raises an important question: are dead fish actually a more important predictor of eDNA than live fish? An alternative explanation could be that the timing of death is simply correlated with the timing of spawning; an act that features the release of genetic material in to the environment. However, based on typical behavior of Hansen Creek sockeye this seems unlikely. With some variation among individuals and mating strategies, and between sexes, Hansen Creek fish begin spawning approximately two days after arrival in the creek and females complete spawning within 3–4 days (McPhee and Quinn, 1998) but males continue to seek breeding opportunities as long as they live (Carlson et al., 2009). Fish of both sexes tend to die about 7–14 (mean = 11) days after they enter the stream if not killed by bears or other forms of premature mortality (Doctor and Quinn, 2009). Because of the short delay between stream entry and spawning, and relatively long post-reproductive life-span, spawning activity peaks well before the live count in Hansen Creek (Quinn, 2005). As Fig. 1 shows, the peak in live count occurred prior to the eDNA peak (2–3 days) and well before the peak in dead (7–9 days) for all sites. Thus, the timing of spawning activity and natural deaths are not well correlated. So, while spawning certainly contributes to the variance in eDNA unexplained by our model, it does not seem a likely explanation for the unexpectedly large influence of dead fish. This issue requires further research, particularly because in most salmon streams, dead fish accumulate whereas they were removed each day during our study. If carcasses are indeed the most important source of eDNA, a strong, cumulative signal from the dead may mask finer scale eDNA dynamics, and indeed change the nature of what is being measured by eDNA sampling (i.e., present vs. cumulative abundance).

Nevertheless, our findings demonstrate that a substantial proportion of variation in salmon eDNA was predicted by the number of fish present, and reversing the model to predict abundance from eDNA should therefore be possible. The relative shedding rates of live and dead fish can be addressed experimentally, or avoided by sampling during migration rather than spawning, while site-specific impacts could be reduced by using a spatially-standardized sampling scheme. Thus, despite remaining uncertainty in some aspects of the abundance-eDNA relationship, our results suggest that estimating fish abundance using eDNA is a realistic goal.

5. Conclusion

As eDNA sampling has become a more common way of surveying aquatic and marine ecosystems, analytical and bioinformatic techniques have improved in step (e.g., Caporaso et al., 2010; Ficetola et al., 2015; Lahoz-Monfort et al., 2016; O'Donnell et al., 2016). Yet, field-based studies have only begun to test the boundaries of genetic sampling as a useful tool for ecology and for applied environmental science (Spear et al., 2015; Yamamoto et al., 2016). Quantitative applications of eDNA sampling could greatly expand the role of these technologies in research, management and conservation, and our results represent an important step in understanding the relationship between species abundance and the amount of genetic material ultimately detectable in the environment. We found that, after accounting for local environmental and biological conditions, eDNA concentrations closely mirrored salmon abundance in our study system. Our results are consistent with others from a variety of marine and freshwater habitats suggesting that eDNA signals degrade quickly over space and time, and this work in a dynamic freshwater environment is an important case study as a coherent view of eDNA processes begins to come into focus.

Acknowledgements

We thank Blakely Adkins, Catherine Austin, Jackie Carter, Jason Ching, Susan Harris, Anne Hilborn, Matt Hilborn, Katie McElroy, Joe Smith and others for the often tedious field work required for this study, and Carl Ostberg for genetics assistance. We also appreciate the valuable input from Mathew Laramie and three anonymous reviewers on previous versions of this manuscript. We acknowledge funding from the IGERT Program on Ocean Change, USGS Ecosystems Mission Area and the University of Washington's Alaska Salmon Program. Use of trade names is for descriptive purposes only and does not constitute endorsement by the U.S. Government.

Appendix A. Design of a Sockeye salmon specific assay and validation

Sockeye salmon tissue samples (n = 5) were sourced from the Elwha river, Washington, USA. These samples were provided to us by biologists at Olympic National Park. We used DNeasy Blood and Tissue Kits (Qiagen) to extract DNA from fin clip tissues.

For marker discovery we sequenced 1162 bp of the mtDNA gene *cytochrome c oxidase III* (CO3) using the Primers CO3F2; 5′-TCAGGCACTGCA GTCTGATT-3′, CO3_tRNA_Arg_R2; 5′-CTTTTGAGCCGAAATCAAGG-3′ and the internal primer tRNA_Gly_IR2; 5′-TTAACCAAGACCGGGTGATT-3′. PCR amplifications were performed in 20 µl reaction volumes, consisting of 10 ng genomic DNA, 1 × Reaction Buffer (Bulldog Bio, Rochester, NY, USA), 200 µM each dNTP (Bioline, Taunton, MA, USA), 50 nM of each primer, and 1.0 units of BioReady rTaq (Bulldog Bio). Cycling conditions consisted of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 1 min and 72° for 1 min 30 s. PCR products were sequenced using a 3730xl DNA analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Sequenced PCR amplification products were edited and aligned using SEQUENCHER v.4.10.1 (Gene Codes Corporation). Regions with species-specific markers were identified for qPCR primer and probe design using MEGA6 (Tamura et al., 2013). Sequences were submitted to GenBank with accession number KU872726.1.

We intended to design a sockeye salmon assay that would be robust enough for application throughout its range. In order to optimize specificity of oligonucleotide hybridization of SECO3_861-930, we choose regions which maximized the number of base-pair mismatches among common co-occurring non-target species. Following Wilcox et al. (2013), we looked for primer sites with mismatches near the 5′ end and in probe-binding sites near the 3′ end. To ensure that SECO3_861-930 would not produce false positives, we tested for specificity against co-occurring salmonid and non-salmonid species (Table A1). All samples were provided to us by biologists at several agencies (Washington Department of Fish and Wildlife, Olympic National Park, National Oceanic and Atmospheric Administration and USGS). 10 pg and 100 pg of genomic DNA from the non-target fish were used for each of the specificity tests, which used the same qPCR parameters as stated in Section 2.2.2. In addition, primer and probe sequences were subjected to NCBI BLAST analysis to test for specificity. To confirm that SECO3_861-930 was valid for our study site sockeye population, we ran Hansen creek samples (n = 5) along with Elwha river samples (n = 5) to compare qPCR Ct values. We ran reactions (2 replicates each) with four different template DNA concentrations (Table A2).

Table A1
Validation of the primer specificity with potential co-occurring.

Non-target species	Ct value with 10 pg template DNA	Ct value with 100 pg template DNA
Species	(Mean ± SD)	(Mean ± SD)
Sockeye salmon (*Oncorhynchus nerka*)	29.92 ± 0.065	26.07 ± 0.019
Chinook salmon (*O. tshawytscha*)	X	35.14 ± 2.232
Coho salmon (*O. kisutch*)	X	X
Chum salmon (*O. keta*)	X	X
Pink salmon (*O. gorbushca*)	X	X
Rainbow trout (*O. mykiss*)	X	X
Coastal Cutthroat (*O. clarkii clarkii*)	X	X
Bull trout (*Salvelinus confluentus*)	X	X
Brook trout (*S. fontinalis*)	X	X
Three-spined stickleback (*Gasterosterus aculeatus*)	X	X
Eulachon (*Thaleichtys pacificus*)	X	X
Surf smelt (*Hypomesus pretiosus*)	X	X
Redside shiner (*Rishardsonius baleatus*)	X	X
Torrent sculpin (*Cottus rhotheus*)	X	X
Reticulate sculpin (*C. perplexus*)	X	X
Pacific lamprey (*Lampetra tridentata*)	X	X

X, no amplification. Ct values are the mean of 6 replicate reactions run for each test.

Table A2
Validation of SECO3_861-930 with Hansen creek sockeye samples.

	1 ng	100 pg	10 pg	1 pg
Elwha River sockeye	22.911 (± 0.281)	26.275 (± 0.248)	30.359 (± 0.166)	34.022 (± 1.003)
Hansen creek sockeye	23.224 (± 0.194)	27.004 (± 0.147)	30.91 (± 0.231)	33.943 (± 0.661)

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