A Note on Sampling Chironomids for RNA-Based Studies of Natural Populations that Retains Critical Morphological Vouchers

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Abstract

The rapid uptake of transcriptomic approaches in freshwater ecology has seen a wealth of data produced concerning the ways in which organisms interact with their environment on a molecular level. Typically, such studies focus either at the community level and so don't require species identifications, or on laboratory strains of known species identity or natural populations of large, easily identifiable taxa. For chironomids, impediments still exist for applying these technologies to natural populations because they are small-bodied and often require time-consuming secondary sorting of stream material and morphological voucher preparation to confirm species diagnosis. These procedures limit the ability to maintain RNA quantity and quality in such organisms because RNA degrades rapidly and gene expression can be altered rapidly in organisms; thereby limiting the inclusion of such taxa in transcriptomic studies. Here, we demonstrate that these limitations can be overcome and outline an optimised protocol for collecting, sorting and preserving chironomid larvae that enables retention of both morphological vouchers and RNA for subsequent transcriptomics purposes. By ensuring that sorting and voucher preparation are completed within <4 hours after collection and that samples are kept cold at all times, we successfully retained both RNA and morphological vouchers from all specimens. Although not prescriptive in specific methodology, we anticipate that this paper will assist in promoting transcriptomic investigations of the sublethal impact on chironomid gene expression of changes to aquatic environments.

Introduction

Understanding the way in which genes and organisms interact with the environment is central to many areas of fundamental and applied biology. Recent advances in DNA and RNA sequencing technologies have driven an explosion of interest in functional studies of such interactions (Pauls et al. 2014, Hughes et al. 2014). Of increasing importance is the way in which these interactions occur in response to anthropogenic change to ecosystems (e.g., Hoffman and Willi 2008, Marchand et al. 2013). In freshwater systems, emerging research is demonstrating the utility of genomic (DNA-based) and transcriptomic (RNA-based) approaches to assessing sublethal impacts of ecosystem degradation on natural populations (Pujolar et al. 2012). In particular, transcriptomics can be used to explore differential expression patterns among populations or species that experience different stressors (both in a lab-based ecotoxicological setting and in natural habitats) and make associations between genes and environmental factors (e.g., Altshuler et al. 2011, Piña and Barata, 2011, Wang et al. 2012). Such research, aimed initially at fundamental biological questions, may allow development of functional molecular proxies in key species to detect and track sublethal adaptive shifts that occur in response to changes to ecosystems – the so-called 'ecotoxicogenomic' approach (Snape et al. 2004) – and will extend and complement current aquatic biomonitoring practices (e.g., Hoffmann and Willi, 2008, Kim et al. 2011, Connon et al. 2012, Tsai and Sung, 2013).

Field sampling of many aquatic macroinvertebrate groups, including chironomids, necessarily involves collection of immature stages (larvae), and thus molecular studies at the species level can be limited by difficulties in making accurate taxonomic identifications. This is due to both the small size of immatures for many groups and lack of diagnostic morphological characters visible under low magnification. For such taxa, accurate diagnoses are impossible without preparation of morphological voucher specimens, which often involves slide-mounting body parts in tissue-clearing fluids and visualisation under high magnification. This represents a significant problem when both molecular and morphological vouchers need to be retained. For example, in DNA-based studies of chironomids, preservation of bulk samples in high purity ethanol and secondary sorting using room temperature ethanol is commonplace. Once tissues

are preserved in ethanol, DNA is stable and excision and slide-mounting of taxonomically critical head capsules as morphological vouchers can be conducted without time constraint. Storage of the remainder of the larval body in high strength ethanol for use in molecular protocols, with unique specimen codes that connect morphological and molecular vouchers 1:1, has been highly successful (e.g., Krosch et al. 2011, 2012). Recent techniques developed for use with pupal exuviae (Krosch and Cranston, 2012), and adopted for use with whole pupae and adults (Krosch and Cranston, 2013, Krosch et al. 2015), utilise whole individuals for non-destructive DNA extractions and retain cuticle intact for voucher preparation post-extraction. The use of DNA barcoding to identify species can alleviate some of these difficulties and remove the need for retention of morphological vouchers in some well-known groups; however, this approach is still limited in some groups where connections between barcodes and morphological taxonomy are lacking. Thus, RNA-based research concerning natural populations of larval chironomids presents new problems for sample storage, voucher preparation and species identification for these invertebrate groups.

RNA degrades much more rapidly than DNA, so storage of specimens in ethanol may not be sufficient to maintain RNA integrity even in the short-medium term (hours-days). Although preservatives exist that are more appropriate for RNA (e.g., RNA*later*®, liquid nitrogen), they are not broadly suitable for initial preservation of chironomids. This is because collecting methods for chironomid larvae are necessarily time-intensive, normally involving collection of a bulk sample (possibly size-sorted using sieves) that contains detritus and non-target taxa, followed by secondary sorting for target taxa under low magnification, voucher preparation of particular body parts from target specimens and confirmation of diagnosis under higher magnification. For studies focused on a single taxon, rather than a whole community, this secondary sorting and species identification to exclude non-targets is essential. Furthermore, post-extraction species diagnosis – either via barcoding (by co-extracting DNA or using bioinformatic approaches on resulting transcriptome data) or retention of cuticle for morphology as described above – may not be feasible for RNA studies of chironomids because RNA extraction techniques are often destructive (e.g., involving crushing of tissues in liquid nitrogen) and multiple specimens may need to be pooled in a single extraction to obtain sufficient RNA quantity for subsequent uses (e.g., cDNA library preparation for high-throughput sequencing).

Taken together, RNA-based studies of natural populations of chironomids clearly require a defined protocol for storage, sorting and vouchering of specimens that both maintains RNA integrity suitable for subsequent processes and retains gene expression profiles as close to natural as possible. Here, we describe such a protocol that extends from stream site to RNA extraction. This protocol was developed and optimised for three Australian species of the chironomid genus *Cricotopus* Wulp (Chironomidae: Orthocladiinae) as part of a broader assessment of differential expression between streams of varying human impact. Development of protocols that facilitate use of RNA-based techniques on natural freshwater macroinvertebrate populations is expected to open up a wealth of novel research areas.

Materials and Methods

Sample collection and transport

Australian species of *Cricotopus* inhabit diverse freshwater ecosystems and some species tolerate ecosystem degradation (Drayson et al. 2015, Krosch et al. 2015). Multiple collections of *Cricotopus* larvae were made from two locations in southeast Queensland, Australia, throughout 2014 and early 2015 (Table 1). Three species were recorded (*C. draysoni* Cranston & Krosch, *C. albitarsis* Drayson, Cranston & Krosch and *C. parbicinctus* Drayson, Cranston & Krosch); the presence and abundance of each varied through the year, with each species generally present at both sites during the same time periods. These species were chosen based on a parallel project on *Cricotopus* systematics led by the senior author that confirmed species diagnoses for all three taxa by associating DNA barcode data with morphological vouchers and found no evidence for cryptic lineages within any of the three species at either location (Drayson et al. 2015, Krosch et al. 2015). Collections involved kick-sampling in riffle sections using a 0.9mm x 0.3mm funnel-tapered polyester sweep net for 30-45 minutes depending on availability of suitable microhabitat. When moving through the stream to subsample in different sections, care was taken to ensure net bag remained submerged and thus not expose specimens to open air which may potentially affect gene expression. Total net samples were strained firstly through a coarse grade (~1mm) then a fine grade (~0.2mm) sieve to remove coarse particulate organic matter and larger invertebrates whilst retaining chironomid larvae (with detritus and non-target organisms). Bulk

samples were transferred to 100mL plastic bottles and immediately fixed with cold absolute analytical reagent (AR) grade ethanol (transported to the sample site on ice). This both euthanases organisms and captures gene expression as close as possible to the point of removal and is a critical step for differential expression studies.

Secondary sorting was conducted as soon as possible but always less than two hours after collection, under low magnification, in a small sorting tray (113mm x 86mm x 18mm) to separate out putative target chironomid larvae: the rest of the bulk sample was held in a 4°C refrigerator and sorting trays were successively filled, sorted and non-target material discarded. Generally, secondary sorting took 2-3 hours. All target specimens were transferred from sorting trays immediately into RNA*later*® (Ambion, Life Technologies, USA) in a glass well on ice. Once in RNA*later*®, RNA fragments are significantly more protected from degradation than in ethanol, but cold storage remains crucial.

Morphological voucher preparation

Morphological vouchers of larval head capsules were prepared immediately on completion of secondary sorting. This involved dissection of the head capsule from the body, using fine-tipped forceps on clean microscope slides (never dissecting two larvae on the same part of a slide), before placement of individual dissected heads in single drops of Hoyer's mountant (van der Meer, 1977) on microscope slides. Immediately as each head was dissected and placed in mountant, larval bodies were transferred to individual RNase-free tubes containing 0.2mL RNAlater® on ice and labelled with codes that related individually and uniquely to each vouchered larval head. Head capsules in mountant were incubated at room temperature for 5 mins to allow mountant solution to rehydrate the head capsule, before compression under a 12mm diameter circular cover slip. Once voucher preparation was complete (which, for a single specimen, takes only 6-7 minutes including incubation), all tubes were transferred to a -20°C freezer until transport on ice to the Molecular Genetics Research Facility (MGRF) at the Queensland University of Technology (QUT).

RNA extraction

On arrival at the MGRF, larval bodies were transferred to a -80°C freezer until use in RNA extraction, while slide vouchers were examined and species identifications recorded against each unique voucher code. Larval body samples of confirmed target species were then selected from the frozen collection. Initial RNA extraction trials (using three and five pooled individuals of *C. draysoni* or *C. albitarsis* or 12 individuals of *Rheocricotopus sp.*) were conducted to determine if and how many individual larvae needed to be pooled into a single extraction to return sufficient RNA. All extractions were conducted in a dedicated RNA fume hood (except for the tissue lysis step) and used dedicated pipettes, sample racks, centrifuge, and filter tips. Prior to commencing extractions all surfaces were cleaned first with 70% AR grade ethanol (diluted with RNase-free water) then with RNase*Zap* (Life Technologies) to remove any remaining RNase contamination. Extractions were conducted following a standard guanidine isothiocyanate-phenol-chloroform protocol (Simms et al. 1993), with some modifications. Briefly, selected larval bodies were thawed on ice and transferred to fresh RNase-free 1.5 mL microcentrifuge tubes containing 1 mL TRIsure™ (Bioline, Australia) or TRIzol® (Life Technologies, USA) and a single 5 mm stainless steel bead (Qiagen, Australia). Tubes were sealed with Parafilm® (Sigma Aldrich, USA) and shaken in a TissueLyser II (Qiagen) at 30Hz for 3 mins. Samples were transferred to new RNase-free tubes and stainless steel beads discarded, as tubes that retain beads can burst during the first centrifugation step (14000rpm/20000g for 15 mins). The RNA phase was separated using chloroform and RNA pellets were precipitated with isopropanol and cleaned with 70% ethanol. RNA was resuspended in 50 µL DEPC-treated water (Bioline) and all samples were visualised by agarose gel electrophoresis (50-100 mL 2% w/v agarose gels with 1-2 µL added GelRed, imaged under UV transillumination) to assess extraction success and detect signatures of degradation and DNA/protein contamination. RNA quality and DNA/protein contamination was also assessed, along with quantification of total RNA, using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and the RNA 6000 Nano kit for total RNA following manufacturer's guidelines. Total RNA was then sent to the Australian Genomics Research Facility (AGRF, Melbourne) for cDNA library preparation and high-throughput sequencing on an Illumina Hiseq2500 using either 100bp paired end or 50bp single end read chemistry as part of a broader parallel study (unpublished data).

Results

The described technique, which was designed specifically to alleviate difficulties in collecting wild chironomid midges for transcriptomic studies, has been used successfully for a total of 40 separate RNA extractions (Table 1). The number of extractions actually represents the sum of 192 individ-

ual chironomid larvae because RNA extractions were conducted on 2-6 individual larvae pooled as a single sample. Moreover, over the course of this research, ten field collections we conducted which resulted in a total of >400 putative target specimens that were preserved and vouchered using the described protocol and include both non-target species and additional unused specimens. It is expected that these samples, currently stored in a -80C freezer, remain valuable for future studies. The protocol, excluding RNA extraction, can be completed by a single person in less than a day: field collection of 30-45 minutes (excluding travel time), secondary sorting of 2-3 hours, voucher preparation 1-2 hours (depending on the number of putative target taxa collected). All morphological vouchers of larval head capsules showed that diagnostic characters (e.g., menta, mandibles, pigmentation) were retained intact, allowing identification using existing species-level keys (designed by the

Table 1. Summary of *Cricotopus* samples used for RNA extraction and resulting template concentration. * indicates samples that formed part of the initial trial extractions. [1]Superscript numbers indicate samples that appear in the agarose gel in Figure 1a and the lane number they were loaded in. ^ indicates the sample for which the exemplar Bioanalyzer plot is provided in Figure 1b.

Site	Species	No. pooled specimens	Concentration (ng/uL)
Cedar Creek	*C. albitarsis*	3	105*
	C. draysoni	5	126*
	C. draysoni	5	341*
	C. draysoni	6	50
	C. draysoni	5	422
	C. draysoni	5	183
	C. draysoni	5	275
	C. draysoni	5	32
	C. draysoni	4	190
	C. draysoni	4	51
	C. draysoni	3	8
	C. draysoni	4	446[8]
	C. draysoni	4	268[9]
	C. draysoni	6	542[10]
	C. draysoni	6	556[11]
	C. draysoni	6	480[12]
	C. parbicinctus	6	62
	C. parbicinctus	6	120
	C. parbicinctus	6	450
	C. parbicinctus	6	57
	C. parbicinctus	6	289
North Pine River	*C. albitarsis*	2	1132
	C. draysoni	5	113
	C. draysoni	5	111
	C. draysoni	5	157
	C. draysoni	5	147
	C. draysoni	5	391
	C. draysoni	5	749
	C. draysoni	5	98
	C. draysoni	5	410
	C. draysoni	5	143
	C. draysoni	4	30
	C. draysoni	4	34
	C. draysoni	6	295[3]^
	C. draysoni	5	130[4]
	C. draysoni	5	260[5]
	C. draysoni	2	58[6]
	C. draysoni	3	227[7]
	C. parbicinctus	5	482
	C. parbicinctus	5	262

senior author, published in Drayson et al. 2015). Identifiability of RNA*later*-preserved specimens did not differ from other *Cricotopus* specimens collected into ethanol/isopropanol for parallel projects (Drayson et al. 2015, Krosch et al. 2015). This demonstrates that storage of larvae in RNA*later*®, at least in the short-medium term, does not cause significant and irredeemable impact on the head capsule cuticle, provided they are incubated for a short period in mountant prior to application of and compression with the coverslip.

All attempted RNA extractions were in some way successful, and pooling of different numbers of individuals did not correlate with resulting RNA concentration. Initial trials suggested that three or five individuals pooled would produce >100 ng/uL RNA (Table 1), whereas 12 individuals, albeit from a different genus, resulted in a relatively lower yield (50 ng/µL). Of the remaining samples, extractions from two pooled individuals gave a range of 58-1132ng/uL, whereas six pooled individuals gave 50-556ng/uL. In this particular case, a total RNA concentration of >100ng/uL was advised (AGRF sample submission guidelines), thus most extractions were conducted on five or six pooled individuals. Exceptions to this occurred where sample sizes were limited for a given collection and biological replicates (for differential expression analyses) were preferred over increasing RNA yield.

Regardless of concentration, resulting total RNA was consistently of high quality (Fig. 1). We provide only exemplars of RNA extraction quality control results for brevity and because all 40 samples are essentially similar, but all results can be made available on request. Agarose gel electrophoresis (Fig. 1a) showed that all samples possessed a strong 18S/28S rRNA band, minimal short (~100 bp) RNA fragments and no high molecular weight gDNA band. Likewise, the exemplar Bioanalyzer plot (Fig. 1b) shows no evidence of DNA/protein contamination (which would otherwise be indicated by the presence of very long fragments) or of RNA degradation (short fragments). Unfortunately, RNA Integrity Numbers (RIN – Schroeder et al. 2006), a standard metric

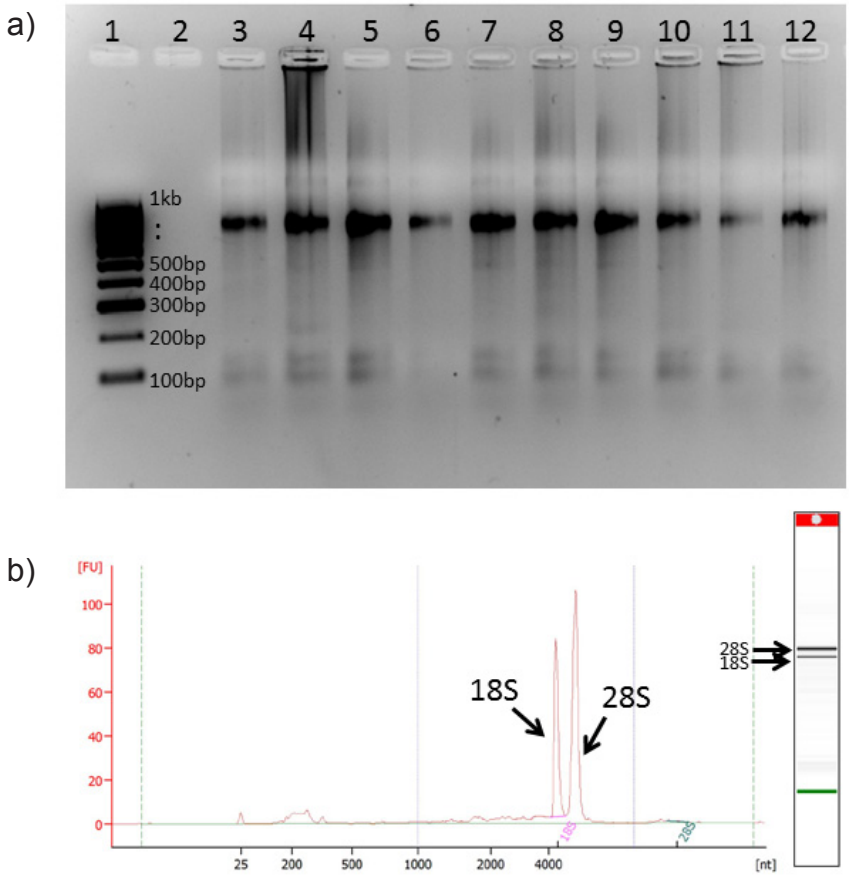

Figure 1. Exemplar RNA extraction QA/QC assessments from the 42 extractions conducted. a) ten extractions analysed using 2% w/v agarose gel electrophoresis: lane 1 contains 1 µL Hyperladder IV (Bioline), lane 2 was intentionally blank, samples are from lanes 3-12; b) exemplar Agilent 2100 Bioanalyzer plot of fragment lengths (x axis) against fluorescence units (y axis). The software automatically attempts to identify the 18S and 28S peaks (labelled below the peak trace in smaller font on an angle) to calculate a RIN.

of RNA quality, could not be calculated reliably for these samples because their peak traces, as interpreted by the Bioanalyzer, were atypical. This most likely related to the software misplacing the 28S peak (e.g., Fig. 1b), possibly driven by differences between the RIN model's expected length of the 28S fragment (based on model eukaryotes) and that observed in *Cricotopus*. Although this can be emended manually, it was not critical to subsequent sample processing. Nevertheless, in general all samples appeared to fit the expected peak trace for samples of RIN 8-10, indicating RNA of high integrity (Schroeder et al. 2006).

Discussion

The expansion of possible RNA-based studies concerning freshwater ecosystems to incorporate small-bodied immature insects that are difficult to identify under low magnification surely will enhance the field by improving our understanding of how ecosystem change impacts taxa. Currently, many studies rely either on laboratory-based ecotoxicogenomic studies of monoculture lab strains (e.g., Li et al. 2009, Planello et al. 2010, David et al. 2012) or on natural populations of larger-bodied, more easily identified species (e.g., Pujolar et al. 2012, Schulteis et al. 2014). A major impediment for research on small aquatic macroinvertebrates like chironomids is the necessity to maintain RNA integrity, 'true' gene expression profiles and morphological vouchers, with sample processing time and storage conditions the most critical factors. The greatest benefit of the described technique, therefore, is to complement and extend field-based ecotoxicogenomic research to incorporate a greater diversity of aquatic macroinvertebrates and explore fully the varied responses of such taxa to ecosystem change.

The key improvements or changes to existing sampling protocols that are critical for maintaining RNA when sampling chironomids can be summarised as follows:

1. When moving through the stream to subsample in different sections, ensure net bag remains submerged and thus does not expose specimens to open air which may potentially affect gene expression.
2. Minimise the time between collection and secondary sorting, and transport samples as cold as possible between collection site and laboratory. It is possible secondary sorting could be conducted at the collection site using a car-powered refrigerator/freezer and portable microscopes. Secondary sorting is best completed within hours of collection to avoid RNA degradation.
3. During secondary sorting and voucher preparation keep sorted target specimens cold, preferably on ice. Ensure vouchering protocol minimises cross-contamination risk.

Although this paper describes an optimised protocol that is likely, with minor adjustment, to have wider applicability across many macroinvertebrate groups and sampling scenarios, this paper does not intend to be prescriptive. There are several alternative options for various steps of the procedure that deserve exploration. Firstly, preservation of bulk samples at the collecting site could conceivably involve transferring the whole sample into RNA*later*® or liquid nitrogen (LN). However, using RNA*later*® for storage of the bulk sample would be expensive and increase the risk of retaining contaminant (non-target) RNA. LN is perhaps most ideal for snap-freezing and storing specimens for RNA work; however, LN possesses considerable health and safety risks when transported into the field (e.g., requiring separation from the main cab of a vehicle, appropriate signage). This can make LN impractical for remote study sites (although it is easy to refill LN dewers 'on the road'). Moreover, subsequent thawing of samples for secondary sorting may still degrade RNA. On the other hand, samples could conceivably be kept alive using water from the site and small aquarium pumps and aerators. Although this method would retain RNA intact while specimens were alive, this may be highly inappropriate for differential expression studies as expression levels may change for some environment-associated genes. Whether this alternative is suitable or not would depend on the system under investigation. For example, heavy-metal enriched stream water will still be high in heavy metals in a sample container and so expression of genes associated with those conditions probably will not change; however, hypoxic water may change in dissolved oxygen content under aeration and thus gene expression associated with hypoxia may change).

Secondly, once bulk stream samples have been secondarily sorted and putative target specimens selected out into cold RNA*later*®, specimens could be transferred to a single vial of RNA*later*® for longer-term storage or transport before morphological vouchers are prepared. However, this risks potential contamination of specimens resulting from storage in multi-species vials, RNA integrity may be reduced by multiple freeze-thaw cycles, and cuticle becomes brittle from long-term storage in RNA*later*®, potentially limiting diagnostic qualities of morphological vouchers. Finally, many alternative methods exist for isolating RNA from tissue specimens, including

a variety of commercially available kits designed around silica matrix spin columns. The method described here inherently relies on precise removal of the aqueous layer to minimise DNA and protein contamination, whilst maximising RNA retention. Although we demonstrate this method can achieve this effectively, spin columns are a more simple option for less experienced lab users (albeit more expensive per reaction). Moreover, extraction kits that are optimised for small amounts of starting tissue may alleviate the need to pool specimens in single extractions and would be strongly recommended for species for which comprehensive systematic knowledge is lacking.

The protocols associated with sampling and preservation to minimise contamination whilst retaining RNA integrity, along with vouchering and specimen tracking are of great importance for many macroinvertebrate groups. Not only do unique vouchers allow specimens to be identified prior to molecular work, specimens can be revisited and perhaps obscure or overlooked morphology validated that may explain unexpected molecular patterns. Furthermore, many taxonomic groups are yet to be comprehensively catalogued in existing DNA barcode libraries (e.g., GenBank, BOLD) and thus molecular species identification remains tenuous for such taxa. Taken together, we consider the described approach optimal to minimise such difficulties and facilitate inclusion of additional taxonomic groups in RNA-based research.

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