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Critical Review

IMPLICATION OF MICRORNA DEREGULATION IN THE RESPONSE OF VERTEBRATES TO ENDOCRINE DISRUPTING CHEMICALS

Brooke E. Cameron,† Paul M. Craig,*†‡ and Vance L. Trudeau†
†Department of Biology, University of Ottawa, Ontario, Canada
‡Department of Biology, University of Waterloo, Ontario, Canada

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Abstract: Micro ribonucleic acids (miRNAs) are recently discovered small regulatory molecules that control messenger RNA (mRNA) translation in plants and animals and have been implicated in a variety of hormone-related physiological pathways. Estrogens, thyroid hormones, and gonadotropins are all known to act on miRNA abundance to cause major shifts in cellular activity, physiology, and homeostatic control mechanisms. Research on cancer biology has also recently considered miRNA as therapeutic targets, because the deregulation of specific miRNAs in various tissues has been correlated with tumorigenesis and other carcinogenic responses. Because many pharmaceuticals are considered to be endocrine-disrupting chemicals (EDCs), their effects on miRNAs may be important to our understanding of basic physiological control and phenotypic outcomes of wildlife exposed to EDCs. Presented is a brief overview of the synthesis, control, and action of miRNAs, focusing on endocrine systems. The antidepressant fluoxetine will be used as an example for miRNA studies in aquatic species, one of the few examples in ecotoxicology. Given the mounting evidence that miRNAs are regulated by hormones, a clear need exists to investigate the potential for environmental EDCs to deregulate miRNA expression and action. *Environ Toxicol Chem* 2015;9999:1–6. © 2015 SETAC

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INTRODUCTION

Pharmaceuticals are aquatic environmental contaminants because of their ongoing presence in streams and water sources [1]. Wastewater treatment plants are the main point source of pharmaceuticals in the aquatic environment [2], because many drugs are incompletely metabolized by patients and are not efficiently removed during the water treatment process. Pharmaceuticals are frequently detected at nanogram to microgram per liter concentrations in effluents and water sources across the globe [2–6]. Consequently, organisms living downstream of discharge sites are exposed to these contaminants and their potential negative effects.

Pharmaceuticals in the environment can act as endocrine-disrupting chemicals (EDCs) because they affect brain and endocrine systems, causing complex effects on neurochemistry, physiology, and behavior of exposed animals. As ecotoxicologists assess the risk of pharmaceutical exposure, various biomarkers are being proposed to allow for more efficient profiling of exposures in the wild. Vitellogenin, an egg-yolk protein produced normally in females, is an example of a known estrogen-responsive biomarker that reflects reproductive disruption if detected in serum or liver of exposed male animals [7]. Emerging evidence indicates that, in addition to disruption of these hormone-dependent processes, pharmaceuticals may cause effects through changes in micro ribonucleic acid (miRNA) expression, presenting a potential new collection of biomarkers for exposure effects. The present review provides a brief overview of the synthesis, regulation, and potential mode of action of miRNA, with a focus on fluoxetine exposure in zebrafish (*Danio rerio)* and goldfish (*Carassius auratus*).

* Address correspondence to trudeauv@uottawa.ca
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MicroRNA

MicroRNAs are evolutionarily conserved small noncoding RNA molecules that act as epigenetic regulators by altering messenger RNA (mRNA) translation and stability. Discovered only 2 decades ago by Lee et al. [8] in the intron of a gene, the first miRNA molecule was originally thought to have no known function. A decade later, miRNAs were recognized as a class of noncoding RNA that are part of a complex regulatory network and involved in the fine tuning of cellular processes [9].

Currently an estimated 2500 human miRNAs are available on miRBase [10]. Database collections contain more than 1600 mature teleost miRNAs (Table 1). MicroRNAs regulate mRNA translation in the cell, with an estimated 30% of protein coding genes regulated by miRNAs [11]. MicroRNAs play a role in nearly all aspects of cellular functioning, such as proliferation, migration, and programmed cell death [12–15]. MicroRNAs also have an important role in endocrine signaling, acting on hormone metabolism and intracellular signaling pathways [16]. The deregulation of miRNAs and their actions have been correlated with various disease states such as obesity, cancer, and diabetes [17–20]. The analysis of miRNA action and function is a novel yet imperative consideration when investigating the actions of EDCs and other environmental toxicants.

Biosynthesis

Figure 1 summarizes the miRNA synthesis pathway. MicroRNAs are 20 to 22 nucleotides long and are products of RNA enzymatic processing steps. Initially, RNA polymerase II transcribes a large precursor molecule, primary miRNA, from intergenic and intragenic regions on chromosomes in the nucleus [21]. The primary miRNA precursor is a single long polycistronic primary transcript and is 5′ capped and adenylated after transcription [22]. Often, multiple miRNAs are clustered together on 1 chromosome; thus, a transcribed primary miRNA

Table 1. Identified precursors and mature sequences for Pisces species found in miRBase [10]

Species	Precursor sequences	Mature sequences
Cyprinus carpio	134	146
Danio rerio	346	350
Fugu rubripes	131	108
Hippoglossus hippoglossus	40	37
Ictalurus punctatus	281	205
Oryzias latipes	168	146
Paralichthys olivaceus	20	38
Salmo salar	371	498
Tetraodon nigroviridis	132	109

may contain several miRNA sequences. Each of the miRNA sequences is 60 to 70 nucleotides long and eventually folds to form a stem-loop hairpin structure within the primary miRNA. The stem loops are then recognized and undergo endonucleolytic cleavage by a multiprocessor complex containing Drosha, an RNA-ase III, and its cofactor, a double-stranded RNA, DiGeorge critical region 8 [11]. The cleavage results in free small stem-looped miRNAs termed precursor-miRNAs [23]. Some miRNAs called "mirtrons" act as introns and are spliced from the primary miRNA by the precursor mRNA splicing enzyme rather than by the enzyme Drosha [21].

Correctly spliced precursor miRNAs are then exported from the nucleus into the cytosol by the nuclear export factor exportin-5, which requires Ran guanosine triphosphate for proper precursor miRNA binding [24]. Ran guanosine triphosphate is a small G-protein translated from the Ran gene, and it is important in the regulation of miRNA biosynthesis by activating or inhibiting exportin-5 activity. Total mature miRNA produced in different cell types can be controlled by Ran guanosine triphosphate, because the amount of Ran guanosine triphosphate in the nucleus versus the cytosol dictates Exportin-5 transport activity [24].

Once in the cytoplasm, precursor miRNA undergoes a second enzymatic cleavage by the RNA-ase Dicer, which clips

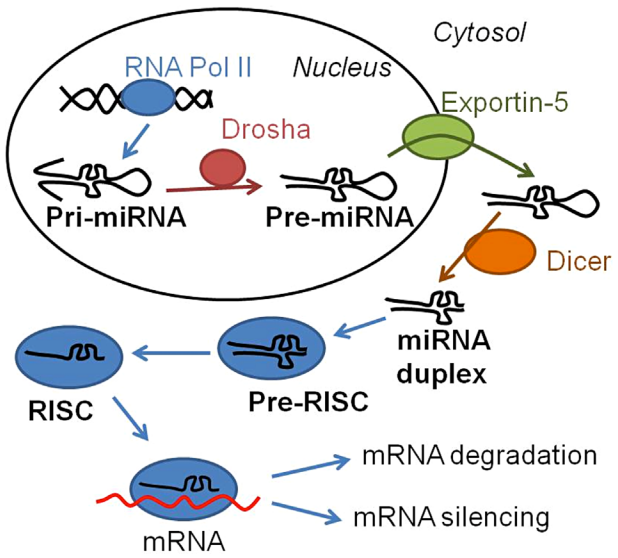

Figure 1. Micro ribonucleic acid biosynthesis pathway. See text for details. RNA Pol II = ribonucleic acid polymerase II; miRNA = micro ribonucleic acid; pri-miRNA = primary miRNA; pre-miRNA = precursor-miRNA; RISC = RNA-induced silencing complex; pre-RISC = precursor RNA-induced silencing complex; mRNA = messenger ribonucleic acid.

the stem loop that connects the 3′ to the 5′ strand of precursor miRNA [25]. This cleavage results in a double-stranded miRNA complex. The duplex miRNA will then bind to Argonaut ribonucleases in an RNA-induced silencing complex, where the 2 strands finally separate into mature miRNA [25]. The less stable miRNA will degrade, and the other will remain in the RNA-induced silencing complex and ultimately bind to and act on mRNA in the cytosol [25].

MicroRNA mechanisms of action

Once properly synthesized, miRNAs act on mRNA targets in 2 major ways: silencing mRNA translation to protein or degrading mRNA molecules. In the first mechanism, miRNAs bind to the 3′-untranslated region (3′-UTR) of target mRNAs to reduce stability or translation [25]. Unlike small interfering RNA, miRNAs do not need to bind perfectly to the mRNA targets. Only a few base pairs, the seed region of 2–8 nucleotides on the miRNA, need to match to block the translational ribosome [26].

Many miRNAs have multiple seed regions in their sequences, thus allowing for them to bind to multiple mRNAs within a cell. This mechanism of binding to mRNA and blocking translation is called RNA silencing; and although the seed region binding is complete, the overall binding is often an imperfect base-pair match. In comparison, when a miRNA binds perfectly or near-perfectly with its target, the mRNA will be degraded. This binding activates Slicer (an Argonaute2 endonuclease), which cleaves the mRNA molecule [16]. Although a report has been made of miRNAs increasing translation and up-regulating mRNAs, most research is on the negative regulation of mRNA by miRNA [14]. Figure 2 gives a summary of miRNA action.

Nomenclature

To differentiate between the thousands of discovered miRNA to date, specific and sequential nomenclature is regularly used (e.g., miR-52). The prefix "mir" is followed by a number based on the order in which it was discovered. The capital R in miR signifies a mature miRNA, and the lowercase r in mir refers to its gene. Letters denote similar mature miRNA sequences (e.g., miR-89a and miR-89b), and suffix numbers denote the same mature miRNA derived from different sections of the genome (e.g., miR-78-1 and miR-78-2). Often, species of origin is also indicated with a 3-letter prefix (e.g., hsa-miR-121 for *Homo sapien* miRNA). MicroRNA is also differentially named when found in specific tissues by using further suffixes.

Regulation and control of miRNA in endocrine systems

Production of miRNA is controlled in 2 major ways: by regulating transcription or by modifying important biosynthesis enzymes, Drosha and Dicer, so that miRNA is not cleaved at all or is not properly cleaved. Within the cell, transcription factors such as SMAD proteins and the effectors of transforming growth factor-β/bone morphogenetic protein have been shown

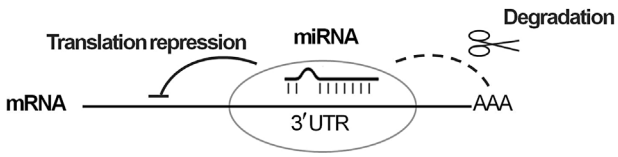

Figure 2. Micro ribonucleic acid (miRNA) action on its messenger RNA (mRNA) target, refer to text for details. UTR = untranslated region; AAA = poly A tail.

to up-regulate miRNA by enhancing the cleavage activity of Drosha in the nucleus [27]. These factors act within the parent cell in which they are produced and can be activated through their regular signaling pathways to modify miRNA and subsequent mRNA activity.

Many studies indicate that hormones are key miRNA regulators. A recent publication by Gupta et al. [11] suggested that estradiol actively controls miRNA production in various tissues such as mammary and ovarian cells. Estrogens regulate miRNA transcription by inactivating RNA polymerase II and regulate precursor miRNA biogenesis by blocking Drosha-mediated processing [11]. This down-regulation of specific miRNA leads to quantifiable changes in proliferation, apoptosis, stress response, transcription, nucleic acid metabolism, mammary gland development, and ovulation, among others.

Gonadotropins also affect miRNA production in ovarian cells. Administration of human chorionic gonadotropin led to an increase in specific miRNA levels in the rat ovary [28]. This increase of specific miRNA resulted in a down-regulation of its target mRNA, which coded for the luteinizing hormone receptor. Exposure of the luteinizing hormone receptor to a preovulatory luteinizing hormone surge has been shown to decrease luteinizing hormone receptor mRNA. Although the mechanisms involved are unclear, the authors suggest that a specific miRNA (miR-136-3p) drives the decrease in luteinizing hormone receptor mRNA and subsequent down-regulation of cell surface luteinizing hormone receptor protein [28]. Discovering the role of miRNA in this and other signaling pathways will lead to an improved understanding of how EDCs affect reproductive function.

Thyroid hormones also affect miRNA expression in mouse liver cells. Specifically, the administration of thyroxine and triiodothyronine in mice results in decreased levels of specific miRNA in mice hepatocytes, resulting in an up-regulation of the miRNA mRNA targets [29]. In comparison, induced hypothyroidism resulted in increased miRNA levels and ultimately down-regulated mRNA targets. This relationship between thyroid hormones, miRNA, and mRNA targets demonstrates an intermediary role of miRNA in thyroid hormone signaling.

EFFECTS OF ENVIRONMENTAL CONTAMINANTS ON miRNAs

MicroRNA and EDCs

Although miRNAs have been at the forefront of biomedical sciences for the past 15 yr, relatively little is known about environmental influences on miRNA. As highlighted in Figure 3, more than 42 000 publications have examined miRNAs related to human health; yet after a more detailed Scopus search of terms, 95 papers have examined miRNAs in relation to toxicology, and only 10 have investigated the effects of EDCs on miRNAs, with only 1 in an aquatic species (zebrafish), by our group [30]. From a toxicological standpoint, more is known about the effects of various physical (silver nanoparticles [31], silica nanoparticles [32]), biological (alcohol [33], Aβ-peptides [34–36]), and chemical (methylmercury [37], tobacco [38], lead [39]) exposures in humans than EDCs, which is surprising given the wealth of research on EDCs and human health (>1700 publications in the past 15 yr; Scopus). Of the limited number of publications, the most recent have identified several EDCs that have a significant impact on miRNA expression and the regulation of breast cancer development in humans. Antifungal agents (fenhexamid, fludioxonil), dichlorodiphenyltrichloroethane, and bisphenol A are all detectable in the environment, are known EDCs, and

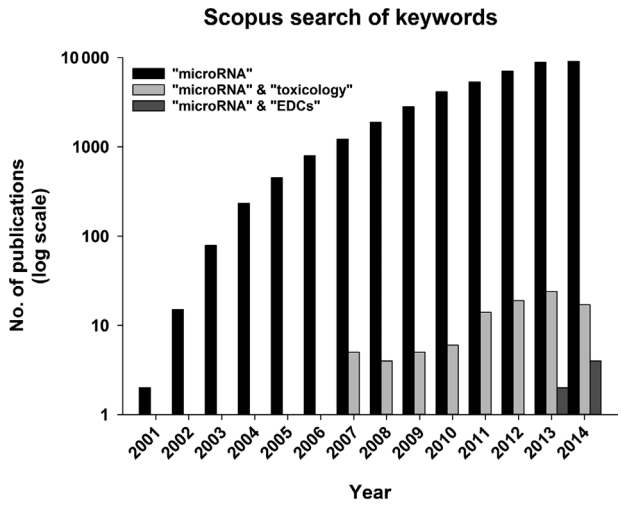

Figure 3. Keyword search using Scopus to identify the prevalence of micro ribonucleic acid (miRNA) in publications for the past 15 yr. Cumulatively, more than 42 000 publications have examined miRNA, although only 95 have examined toxicological effects on miRNA, and surprisingly only 10 have investigated the effects of endocrine-disrupting chemicals on miRNA and the downstream consequences. Only 1 of these 10 examined the environmental influence on an aquatic species (zebrafish [28]). miRNA = micro ribonucleic acid; EDCS = endocrine-disrupting chemicals.

have been demonstrated to up-regulate miR-21, a key miRNA involved in the proliferation of cancer development in breast cancer cell lines [40,41]. Possibly adverse effects mediated by miRNA may occur in nonhuman species. This is particularly relevant to aquatic species, where the effects of environmentally relevant doses of EDCs are well described. This highlights the importance of investigating the effects of EDCs on miRNA, because they may be one of the driving forces behind phenotypes presented after EDC exposure. Likewise, significant potential lies in understanding the transcriptional response of miRNA after chronic, low-level exposures, because these may provide an epigenetic "fingerprint" to environmental contaminants.

MicroRNA and fluoxetine

To our knowledge, only a single pharmaceutical has been considered in relation to effects on miRNAs in an aquatic species. Fluoxetine is the active ingredient in Prozac, a popular pharmaceutical used to treat depression, bipolar disorder, and other psychiatric conditions. Because of its heavy prescription use, incomplete metabolism in humans, and inefficient removal from wastewater, fluoxetine is detectable in aquatic environments downstream of wastewater treatment plants across the globe [42]. Fluoxetine is a selective serotonin reuptake inhibitor and works by blocking the reuptake of serotonin at the synaptic cleft by binding to the serotonin reuptake transporter, thus enhancing the serotonin signal. Fluoxetine is therefore known to disrupt several endocrine systems of fish by acting on the serotoninergic pathways, altering biological systems controlled by serotonin, such as feeding, metabolism, and reproduction [43–46].

Studies in mammals have also found that fluoxetine alters miRNA levels in select tissues, demonstrating a link between miRNA and fluoxetine exposure [47,48]. One study found that the effect of fluoxetine exposure on serotonin reuptake transporter translation is mediated by microRNA-16 (miR-16) in the raphe nuclei of mice [47]. The authors demonstrated that fluoxetine inhibits canonical Wnt signaling in the raphe nuclei,

which normally negatively regulates the primary/precursor miR-16 levels. By inhibiting Wnt signaling, fluoxetine increased miR-16 synthesis and subsequently decreased serotonin reuptake transporter mRNA translation. Conversely, fluoxetine lowered the levels of miR-16 in adjacent nuclei by promoting the translation of the neurotrophic factor S100b in the raphe nucleus. The S100b acts as a paracrine signal to inhibit miR-16 production in the locus coeruleus to promote serotoninergic-specific functions. The effect of fluoxetine on miR-16 in the raphe nucleus also induces serotoninergic neurons to secrete B-cell chronic lymphocytic lymphoma 2, which acts on the hippocampus in mice to reduce miR-16 production and causes an increase in hippocampal neurogenesis [48]. Ultimately, these studies demonstrate the important role of specific miRNAs in the effects of fluoxetine in mammals.

Craig et al. [30] recently explored the effects of fluoxetine on hepatic miRNA in zebrafish, using a custom miRNA microarray to identify potential players in metabolic disruption. After a 7-d waterborne exposure, 6 hepatic miRNAs increased in fluoxetine-exposed female zebrafish (dre-miR-22b, dre-miR-140, dre-miR-210a, dre-mir-301, dre-miR-457b, and dre-let-7d). As determined through target prediction and pathway analysis, each miRNA may interact with dozens of mRNAs associated with metabolism. In general, the miRNAs that were up-regulated were predicted to be responsible for down-regulating pathways such as insulin signaling, cholesterol synthesis, and triglyceride synthesis. In particular, 2 miRNAs, dre-miR-140 and dre-let-7d, were predicted to bind to mRNA subunits of the adenosine monophosphatase (AMP)-activated protein kinase (AMPK) enzyme, a master regulator of metabolism. An inverse relationship was found between the relative transcript abundance of the $\alpha 1$ and $\alpha 2$ subunit mRNAs and dre-miR-140 and dre-let-7d, suggesting that AMPK-related pathways may be compromised by fluoxetine exposure as a result of increased miRNA abundance. One of the miRNA, miR-22, has also been found to play important roles in insulin signaling in other mammalian and fish species such as human (*Homo sapiens*), Japanese medaka, stickleback (*Gasterosteus aculeatus*), Fugu pufferfish (*Takifugu rubripes*), and zebrafish [49]. In humans, overexpression of hsa-miRNA-22 is linked to increased insulin-stimulated Akt phosphorylation in the liver. In other fish species, miR-22 is also predicted to target components of the insulin pathway by targeting phosphoinositide 3-kinase (PI3K) subunits.

We have investigated these 6 miRNAs in goldfish to determine whether the effect of fluoxetine on miRNA abundance is conserved across fish species. Adult female goldfish ($n = 8$) were exposed to environmentally relevant levels of fluoxetine (either 0.5 or 1 μg/L) in a static renewal exposure over 7 d. Goldfish were weighed and randomly placed in 1 of 3 exposure groups, control (0 μg/L fluoxetine), low (0.5 μg/L fluoxetine), and high (1 μg/L fluoxetine), with no weight differences between groups. Fish were kept in 70-L tanks (2 replicates per exposure group with 12 fish per tank) with 80% water renewal and fluoxetine addition every 2 d for 7 d. The abundance of these hepatic miRNAs was quantified by quantitative polymerase chain reaction as reported by Craig et al [30]. Five of the 6 hepatic miRNAs increased significantly in both exposure groups, including miR-22 (Figure 4). These data suggest that EDC exposure may cause similar effects in different fish species. Speculating that up-regulation of these miRNAs would cause significant metabolic disruption in goldfish as in zebrafish is tempting; however, although miRNA

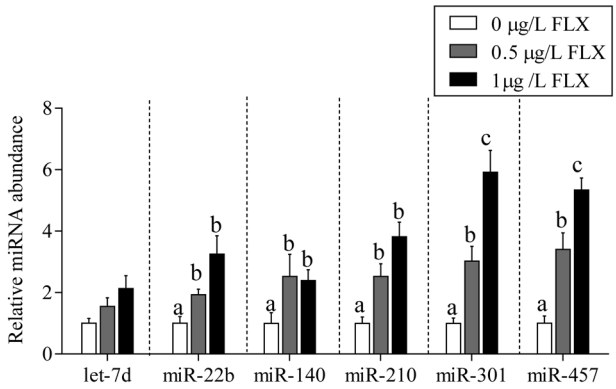

Figure 4. Relative micro ribonucleic acid (miRNA) abundance (mean + standard error of the mean) in female goldfish ($n = 6$) liver tissue of dre-let-7d, dre-miR-22b, dre-miR-140-5p, dre-miR-210, dre-miR-301a, and dre-miR-457b-5p after a 7-d waterborne exposure to 0 μg/L, 0.5 μg/L, and 1 μg/L fluoxetine. Different letters (a, b) indicate significant differences between means (one-way analysis of variance, Tukey's post hoc, $p < 0.05$). Three miRNAs (dre-let-7d, dre-miR-22b, and dre-miR-140) were $\log_{10}$ transformed before analysis to normalize data. The present research was conducted in accordance with the Canadian Council on Animal Care. miRNA = micro ribonucleic acid; miR = mature miRNA; FLX = fluoxetine.

sequences are highly conserved across species, the mRNA targets are not well conserved, or not at all conserved. The 3′-UTR of mRNA is the binding target of miRNA and varies widely between species. As such, a given miRNA may alter different pathways in different species. No complete transcriptome or genome database is available for goldfish, so miRNA targets remain unknown. Nevertheless, clearly in goldfish, waterborne fluoxetine causes reduced feeding and liver function [50] and alterations in the plasma proteome [7], all of which indicate metabolic disruption. The specific link between deregulated hepatic miRNAs and these effects in goldfish remained to be investigated.

CONCLUSIONS

Very few ecotoxicology studies have considered miRNA in contaminant exposure studies (Figure 3), demonstrating a major gap in the scientific literature in this field compared with cancer biology and endocrinology. Water is arguably one of our most precious resources, yet it faces extensive and complex threats in terms of both quantity and quality in the short- and long-term. In North America alone, alarming statistics have indicated a significant increase in the detectable level of EDCs in the Great Lakes over the past 10 yr, to levels well above "environmental concern" (ng/L–μg/L range) [51]. More than 165 pharmaceuticals are detectable in the Great Lakes and the watersheds that feed them [51]. This is troubling, because the impact of these contaminants is unknown for not only the 4000 species of plants and animals that inhabit these waters, but also the 26 million people who rely on the Great Lakes as a source of drinking water [52]. Combining these threats with the evidence that miRNAs are involved in endocrine regulation and disease development in humans, focusing attention on the role of miRNAs in the response of aquatic vertebrates to EDCs is essential. In the present review we confirm that a known EDC (fluoxetine) can significantly increase the abundance of specific miRNAs in 2 cyprinids, enhancing our understanding of the interaction between EDCs and miRNA expression. Downstream functional impacts remain to be elucidated. The present review illustrates the importance of these small molecules in

normal and abnormal physiological function and highlights the need for more ecotoxicological studies examining the functional impact of changes in miRNA abundance.

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Data availability—Data, associated metadata, and calculation tools are available publicly on request from the authors (trudeauv@uottawa.ca).

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