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The ninespine stickleback as a model organism in arctic ecotoxicology

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ABSTRACT

Background: The Arctic is subject to atmospheric deposition of persistent organic pollutants through the process of global distillation. It also contains thousands of sites with local sources of contamination, including military installations, mining operations, and petroleum extraction facilities. Pollutants accumulate in surface waters.

Aim: To investigate the utility of ninespine stickleback (*Pungitius pungitius*) as a sentinel fish species to monitor the presence and biological effects of chemical contamination in the Arctic.

Organism: *Pungitius pungitius* is a circumpolar species that occurs in freshwater, brackish water, and marine habitats. It is often the most abundant fish present. It appears to be relatively hardy with respect to potentially lethal effects of contaminants, which allows investigation of perturbed biological processes such as endocrine, gene expression, and developmental disruption.

Biomarker development: We developed a homologous vitellogenin assay for the ninespine stickleback to assess the presence of estrogen-modulating contaminants in surveys of arctic contaminated sites. We also validated a thyroxine (T_4) assay for the ninespine stickleback to enable investigations of thyroid-disrupting contaminants. We review recent work that demonstrates the efficacy of transcriptional profiling to identify disrupted gene networks by finding functionally related groups of genes that are up- or down-regulated due to contaminant exposure.

Results and conclusions: We collected spatially explicit contaminated site data for the Arctic and found that more contaminated sites lie within the distribution of the ninespine stickleback than within that of the threespine stickleback. This biogeographic advantage combined with a suite of biomonitoring tools makes the ninespine stickleback a suitable model organism for studying contaminants in marine, brackish, and fresh waters throughout the Arctic.

Keywords: biomonitoring, endocrine disruption, *Gasterosteus aculeatus*, *Pungitius pungitius*, sentinel species, stickleback, vitellogenin.

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INTRODUCTION

In the early nineteenth century, scientists believed that only the vital force of living organisms could create organic matter. Friedrick Wöhler (1828) shattered this idea when he accidentally synthesized urea by mixing cyanic acid with ammonia. Wöhler managed to synthesize, from inorganic ingredients, a substance previously believed only made by the liver. The obvious implication, borne out over the subsequent nearly two centuries of chemical experimentation, was that chemists could synthesize just about any naturally occurring organic compound. A less obvious outcome was the synthesis of tens of thousands of chemicals that do not exist in nature. The industrialization of organic chemistry brought great riches to leading states such as Germany and the United States, but also led to the pollution of the biosphere with artificial compounds. Many of these compounds proved to be toxic, persistent in the environment, and subject to long-range atmospheric or oceanic transport; collectively, such chemicals are known as persistent organic pollutants (POPs) (Wania and Mackay, 1996).

The surprising discovery in the 1980s that Inuit women of arctic Canada have substantially higher levels of certain POPs in their breast milk than do women in southern Canada sounded the alarm that the Arctic is the recipient of globally transported contaminants (Dewailly *et al.*, 1989). The chemical structure and hence volatility of POPs vary widely. Many POPs are sufficiently volatile to enter a gaseous phase at high temperatures, but at low temperatures they condense from the atmosphere and are deposited in terrestrial and aquatic systems. These chemicals therefore undergo the process of global distillation with fractionation whereby evaporation exceeds deposition at low latitude and deposition exceeds evaporation at high latitude; hence, they tend to preferentially accumulate at high latitudes with fractionation depending on mobility (Wania and Mackay, 1993, 1996; Blais, 2005). Low temperatures and low-intensity sunlight at high latitudes slow the deterioration of POPs, giving them a long residence time (Wania and Mackay, 1993; Bard, 1999).

Since most POPs are used in the northern hemisphere, the Arctic tends to accumulate more POPs than the Antarctic (Bard, 1999). Furthermore, POPs are often lipophilic and therefore bioaccumulate in individuals and biomagnify in food webs, processes that are especially pronounced in the lipid-rich Arctic (Wania and Mackay, 1993; Bard, 1999). The food web biomagnification of POPs explains the high concentrations found in Arctic Indigenous Peoples such as the Inuit whose traditional subsistence diet consists of long-lived, high trophic level marine animals (Wania and Mackay, 1993; Ayotte *et al.*, 1995). The Arctic also contains thousands of locally contaminated sites, including military installations, mining operations, and petroleum extraction facilities (Figs. 1, 2). The Arctic is warming at more than twice the rate of the global average (Serreze and Barry, 2011; McKinney *et al.*, 2015), which is likely to lead to remobilization of sequestered contaminants from permafrost and ice (Ma *et al.*, 2011; Grannas *et al.*, 2013; Noyes and Lena, 2015). The high rate of erosion occurring along the coastline of the Arctic Ocean and arctic seas, associated with the loss of sea ice, may increase the remobilization of contaminants at the same time that increasing shipping and development cause additional local pollution. Together, these facts and trends indicate a clear need for a tractable fish model for arctic ecotoxicology.

Aquatic habitats accumulate contaminants from their watershed and hence aquatic organisms often suffer disproportionately from the effects of toxicants (Luoma and Rainbow, 2008). Fish ecotoxicology is an important area of study in aquatic systems because fish reflect contaminant levels in their habitat, are consumed by predatory wildlife, and are a critical

human food source (Tierney *et al.*, 2014). Furthermore, many fish species have been utilized as models for ecotoxicology, including for studies of contaminants in natural systems and for human health effects (Tierney *et al.*, 2014). This is due in part to the fact that many fish embryos are transparent, allowing direct visualization of developmental disruption, and because endocrine and genetic systems are both broadly and highly conserved across vertebrates.

One of these model species is the threespine stickleback (*Gasterosteus aculeatus*), which has been shown to have great utility owing to its (1) widespread distribution in marine, brackish, and fresh waters of the northern hemisphere, (2) hardiness with respect to contaminant exposure, (3) ease of maintenance in the laboratory, (4) sequenced genome and numerous molecular tools and validated biomarkers, and (5) many other useful behavioural and physiological traits (Pottinger *et al.*, 2002, 2011a, 2011b, 2013; Katsiadaki *et al.*, 2007, 2012; Sanchez *et al.*, 2008; von Hippel, 2010; Sebire *et al.*, 2011; Jones *et al.*, 2012; Glazer *et al.*, 2015). Because of these advantages, the threespine stickleback has been used to monitor and study a diverse array of contaminants, including perchlorate (e.g. Furin *et al.*, 2013), mercury (e.g. Kenney *et al.*, 2014), polycyclic aromatic hydrocarbons (e.g. Williams *et al.*, 2009), copper (e.g. Santos *et al.*, 2010), phthalates (e.g. Aoki *et al.*, 2011), nanoparticles (e.g. Sanders *et al.*, 2008), and so on. The value of this species for chemical characterization is particularly great for endocrine disruptors (Katsiadaki *et al.*, 2002a, 2002b, 2006, 2010; Hahlbeck *et al.*, 2004; Jolly *et al.*, 2006, 2009; Andersson *et al.*, 2007; Björkblom *et al.*, 2007; Nagae *et al.*, 2007; Sebire *et al.*, 2008, 2009; Aoki *et al.*, 2011), a fact that is reflected in regulatory OECD test guidelines (OECD, 2012). The threespine stickleback, however, is not found throughout much of the Arctic (Fig. 1) and thus has limited utility there. In contrast, the closely related ninespine stickleback (*Pungitius pungitius*) is an arctic-centred species, and shares many of the utilitarian features of the threespine stickleback, giving it the potential to also serve as a model species in biology (Merilä, 2013).

The objectives of this study are to demonstrate the utility of the ninespine stickleback as an arctic biomonitoring species, and to present new tools that we developed to this effect. We first analyse the geographic advantages of arctic investigations of contaminants using the ninespine stickleback, or combining its use with the threespine stickleback. We then review genomic resources for ninespine stickleback ecotoxicology, including tools for transcriptional profiling to identify disrupted gene networks by finding functionally related groups of genes that are up- or down-regulated due to contaminant exposure. We present a homologous vitellogenin assay that we developed to assess the presence of estrogen-modulating contaminants in surveys of arctic contaminated sites. We also present our validation of a thyroxine (T_4) assay for the ninespine stickleback to enable investigations of thyroid-disrupting contaminants. This suite of biomonitoring tools makes the ninespine stickleback a suitable model organism for studying contaminants in marine, brackish, and fresh waters throughout the Arctic.

DISTRIBUTION AND BIOMONITORING

The threespine and ninespine sticklebacks both have distributions throughout much of the northern hemisphere, but the ninespine stickleback is an arctic fish that extends to lower latitudes while the threespine stickleback is a temperate fish that extends into the Arctic (Fig. 1). The ninespine stickleback is often the most abundant fish present in arctic freshwater and brackish water sites (e.g. Byrne *et al.*, 2015).

Using ArcGIS 10.3 (Esri, Redlands, CA), we collected spatially explicit contaminated site data from the US Geological Survey's Alaska Resource Data File (ADNR, 2015), the Alaska

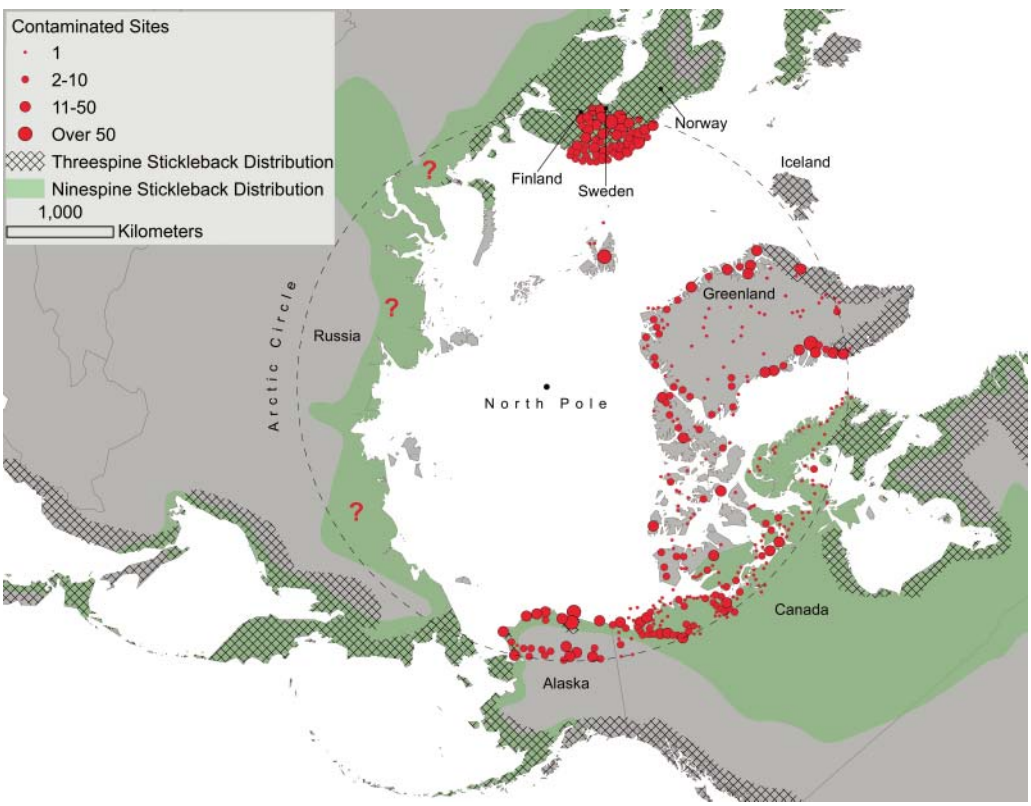


Fig. 1. Distribution of arctic contaminated sites (defined as north of the Arctic Circle, shown by the dashed line) and the ranges of the threespine stickleback (Bell and Foster, 1994) and ninespine stickleback (J. Merilä, unpublished data). Both species also occur in marine waters. Arctic contaminated sites were digitized from a composite database published by Robin des Bois (RdB, 2015). Original sources for the data can be found on the website, as well as links for individual countries. Contaminated sites data are not available for Russia.

Department of Environmental Conservation (ADEC, 2013), the Alaska Community Action on Toxics database (ACAT, 2015), and the Alaska Oil and Gas Association (AOGA, 2015) to map all the known contaminated or potentially contaminated areas in Alaska, and merged them into a single geodatabase to reduce spatial redundancy. We then digitized data from a composite dataset on arctic contaminated sites from a non-profit group (RdB, 2015). Unfortunately, data for Russia were not available from that or any other dataset we examined.

Distributional data for the threespine stickleback were provided by Bell and Foster (1994), with modifications based on collection records in Alaska by the von Hippel lab. The distribution of ninespine stickleback was digitized using information from various published sources (J. Merilä, unpublished). By overlaying the distributions of the ninespine and threespine sticklebacks and the contaminated sites, we found that ~818 arctic contaminated sites lie within the distribution of the ninespine stickleback, while ~585 arctic contaminated sites lie within that of the threespine stickleback (Fig. 1; exact numbers depend on method of counting sites). If data from arctic Russia were available, the

distributional results would be even more biased towards ninespine stickleback because threespine stickleback are not found along much of the Russian coast of the Arctic Ocean (Fig. 1). Therefore, from a purely distributional point of view, the ninespine stickleback is the more valuable species for arctic monitoring; research programmes using both species, however, would result in the most complete geographical coverage.

The value of using both stickleback species is well demonstrated through an analysis of contaminated sites in the Alaskan Arctic, as defined by the US Arctic Research Policy Act (ARPA) of 1984, which includes coastal areas along the Bering Sea and the Aleutian Archipelago. Limiting our overlay to just the arctic regions of Alaska, approximately equal percentages of contaminated sites occur within the range of both species, with a slight advantage to the ninespine stickleback (Table 1). Some contaminated sites in the Alaskan Arctic contain only threespine stickleback [for example, many of the Aleutian Island sites (Kenney *et al.*, 2012; 2014; Kenney and von Hippel, 2014)], while others contain only ninespine stickleback (e.g. much of the Arctic Ocean coastline; Fig. 2).

Analytical chemistry results for ninespine stickleback collected on St. Lawrence Island in the Bering Sea reveal that the patterns of body burdens for numerous POPs closely mirror those of the Siberian Yupik people who live a largely subsistence lifestyle on the island (Byrne *et al.*, 2015). These results indicate that the ninespine stickleback can be effectively used for biomonitoring of POPs for Arctic Indigenous Peoples. Site-based differences in body burdens of ninespine stickleback within the same stream show that this species can be used for biomonitoring even on small spatial scales (Byrne *et al.*, 2015). Furthermore, the ninespine stickleback is hardy with respect to contaminant effects, which allows investigation of perturbed biological processes such as endocrine and developmental disruption.

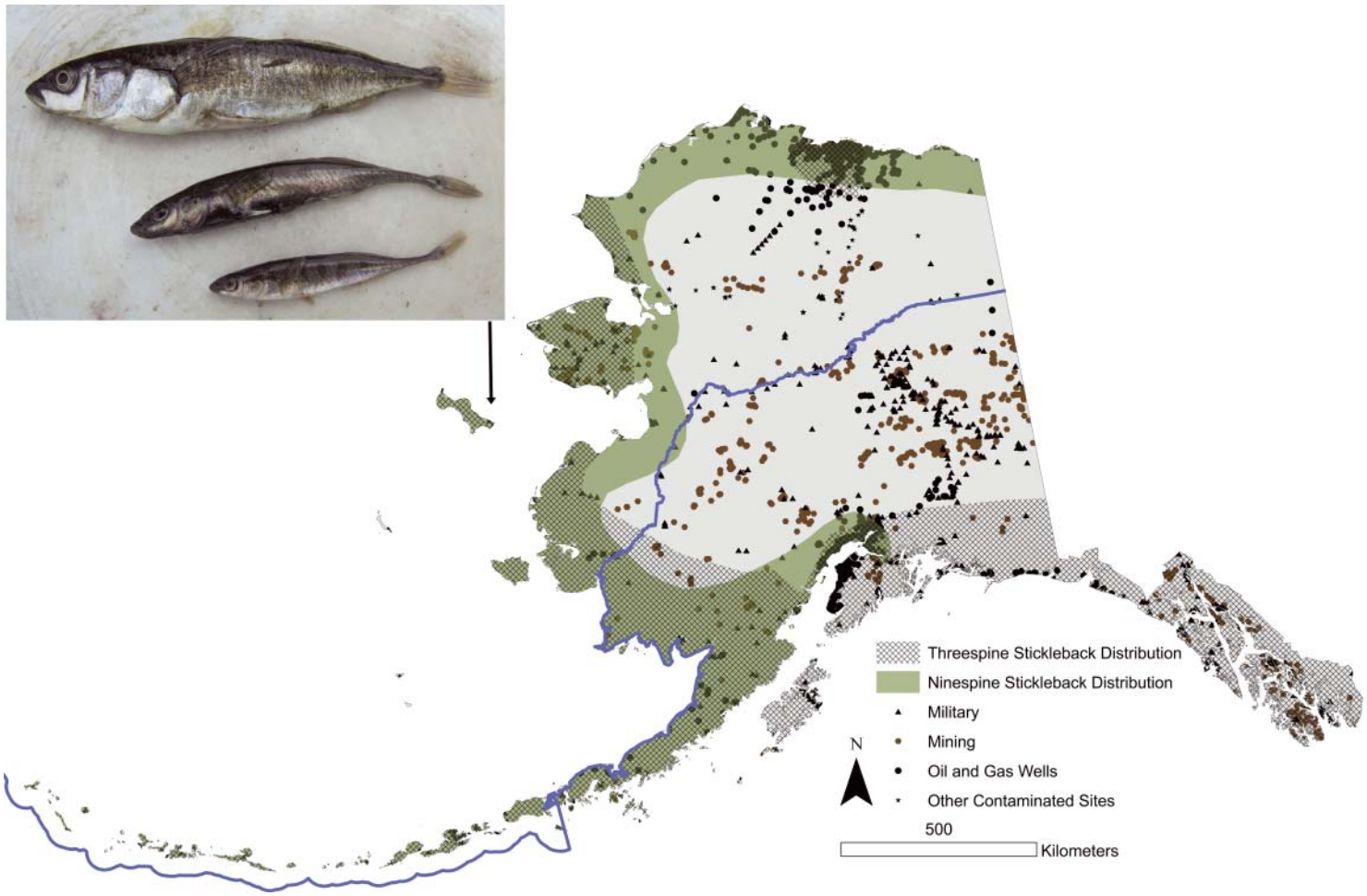
GENOMIC RESOURCES FOR ECOTOXICOLOGY

Recent advances in genomics increase the utility of the ninespine stickleback as a circumpolar sentinel of risks associated with environmental chemicals in at least two ways. First, genomic tools can clarify evolutionary relationships between different *Pungitius* lineages, ensuring that closely related and phenotypically similar congeners are not misidentified

Table 1. Contaminated sites within the distributional range of threespine stickleback and ninespine stickleback in the Alaskan Arctic

Contaminated site category	Number of sites	Percent within threespine stickleback distribution only	Percent within ninespine stickleback distribution only	Percent within both species' ranges
All military	~400	17%	15%	35%
Mining	~190	5%	11%	41%
Oil and gas	~6000	2%	8%	81%
Other	~500	9%	16%	35%
Total	**~7090**	**3%**	**9%**	**74%**

Note: See Fig. 2 for geographic ranges and definition of the Alaskan Arctic. The number of sites per category is a rough estimate based on diverse data sources that may have overlapping information (ACAT, 2015; ADNR, 2015; AOGA, 2015; RdB, 2015). The percentages in each column are mutually exclusive (e.g. the total percent of all contaminated sites within the distribution of the ninespine stickleback = 9% + 74% = 83%).


Threespine Stickleback Distribution
Ninespine Stickleback Distribution
Military
Mining
Oil and Gas Wells
Other Contaminated Sites
N
500
Kilometers

as ninespine stickleback. In addition, because the genetic divergence among different *P. pungitius* populations can be considerable, their sensitivity to toxicants may differ. Hence, knowledge about genetic differentiation may help to explain potential differences in responses to the same contaminants. Second, genomics provides a new suite of approaches and biomarkers for ecotoxicology and thereby advances our understanding of the impacts of harmful chemicals on individuals, populations, communities, and ecosystems.

The taxonomy and systematics of the genus *Pungitius* traditionally relied on morphology and mitogenomic gene fragments (Keivany and Nelson, 2000; Mattern, 2007). However, morphology-based inferences about taxonomy and systematics in this genus can be unreliable (Mattern, 2007; Wang *et al.*, 2015). In addition, ongoing studies based on single nuclear polymorphic markers (SNPs) have identified at least four cases of hybridization between *Pungitius* taxa, and some populations currently recognized as *P. pungitius* may actually be representatives of different species (B. Guo, T. Shikano and J. Merilä, unpublished data). While these hybridization events do not directly involve populations residing in the Arctic, considerable genetic divergence exists even between different arctic populations of *P. pungitius* (Aldenhoven *et al.*, 2010; Shikano *et al.*, 2010). Of particular interest might be the fact that the North American populations, earlier recognized as a different species (*P. occidentalis*), are genetically divergent from the Eurasian populations (B. Guo, T. Shikano and J. Merilä, unpublished data). Hence, this divergence may also translate as differential responses to stressors, and biomarkers obtained with new genomic technologies can help to provide the evolutionary framework with which to compare population responses to toxicants.

New genomic technologies and resources provide a rich and versatile source of biomarkers for ecotoxicology studies. Given the close evolutionary affinity of the ninespine stickleback to the threespine stickleback (Kawahara *et al.*, 2009; Guo *et al.*, 2013), genomic resources available from the latter have been helpful in devising biomarkers and genetic linkage maps for the ninespine stickleback (Shapiro *et al.*, 2009; Shikano *et al.*, 2010, 2011, 2013). However, while the threespine stickleback genome remains a useful resource for ninespine stickleback genetics, 'genome-free' *de novo* approaches such as analyses of restriction site associated DNA markers ['RAD-seq' (Miller *et al.*, 2007)] have started to replace microsatellites and direct sequencing of short gene fragments as biomarkers in ninespine stickleback (Bruneaux *et al.*, 2013). For instance, ultra-high-density linkage maps based on >40,000 SNP markers are now available for the ninespine stickleback (Rastas *et al.*, 2016).

Assessing individual and population level variation in transcript abundance (i.e. gene expression) has become a tool of trade in contemporary ecotoxicology (Snell *et al.*, 2003; Piña *et al.*, 2007). It is now feasible to quantify tissue-specific expression of whole transcriptomes

Fig. 2. Distribution of arctic contaminated sites within the state of Alaska and the ranges of the threespine stickleback (Bell and Foster, 1994; F.A. von Hippel, unpublished data) and ninespine stickleback (J. Merilä, unpublished data). Both species also occur in marine waters. The geographic boundaries of the Arctic shown by the blue line are as defined by the US Arctic Research Policy Act (ARPA) of 1984. *Data sources*: military sites representing current and formally used defence sites (ACAT, 2015); mining sites (data maintained by the US Geological Survey; ADNR, 2015); oil and gas wells (AOGA, 2015); other contaminated sites from Robin des Bois database (RdB, 2015). Inset: Photo of reproductively mature female anadromous threespine stickleback (top), reproductively mature male ninespine stickleback (middle), and immature ninespine stickleback (bottom), all collected near the formerly used defence site at Northeast Cape on St. Lawrence Island (arrow); photo credit: J. Postlethwait.

from any organism using massive parallel sequencing of gene transcripts [i.e. RNA-seq (Ozsolak and Milos, 2011)]. Although *de novo* assembly of transcriptomes is possible, the full power of this approach is harnessed best when sequenced transcripts can be fully annotated by aligning them against a well-annotated reference genome. The threespine stickleback genome provides a fair template for annotation of the ninespine stickleback transcriptome. But many RNA-seq reads are from rapidly evolving untranslated regions (UTRs) of the genome, and the 13 million years of sequence divergence between these two species (Bell *et al.*, 2009) leads to some loss of information and biases in read mapping. To help overcome this problem, a high-quality (average coverage ≈110×) ninespine stickleback whole-genome sequence from a Lake Pyöreälampi (Finland) individual was obtained with single molecule real-time sequencing (Pacific BioSciences), providing a useful resource for ninespine stickleback transcriptomics. The current version of this genome assembly consists of 5322 contigs (N50 contig length = 1.2 Mbp) which sum to 521 Mbp in total length (J. Merilä, unpublished data), compared with the ~460 Mbp in the current genome assembly of threespine stickleback (http:www.ensembl.org/Gasterosteus_aculeatus/Info/Index; Jones *et al.*, 2012).

NINESPINE STICKLEBACK VITELLOGENIN ASSAY

Clear molecular markers for pollutant effects are needed to identify risks. Vitellogenin (VTG) is an egg-yolk precursor protein produced in female liver cells due to induction by ovarian estrogens (Sumpter and Jobling, 1995). Male vertebrates produce insufficient estrogen to stimulate VTG production; hence, VTG expression in males is a commonly used biomarker for exposure to estrogenic contaminants (Sumpter and Jobling, 1995). Environmental estrogens such as many pesticides, PCBs, and plasticizers are linked to a variety of developmental disorders and certain cancers in wildlife and humans (Norris and Carr, 2006). Therefore, arctic ecotoxicology would clearly benefit from a validated VTG assay for the ninespine stickleback, similar to the assay already widely deployed for the threespine stickleback (Hahlbeck *et al.*, 2004). To address this need, we developed and here describe a VTG enzyme-linked immunosorbent assay (ELISA) for the ninespine stickleback. The first step towards development of a homologous VTG ELISA involved induction of this protein by estrogen treatment in ninespine stickleback, followed by isolation and purification of VTG to immunize a rabbit and raise the antibody.

Chemicals, consumables, and equipment

The steroid used for estrogenizing the fish was the potent synthetic estrogen 17α-ethinyl-estradiol (EE_2, E4876, Sigma-Aldrich, Poole, UK). Other reagents used for setting up the VTG assay included anti-Rabbit IgG (whole molecule conjugated to alkaline phosphatase, affinity isolated), alkaline phosphatase substrate (1 $mg \cdot mL^{-1}$ 4-nitrophenyl phosphate (pNPP) in 0.2 M Tris buffer), bovine serum albumin (BSA), and anti-protease cocktail (P2714), all from Sigma-Aldrich (Poole, UK).

Ethanol (HPLC purity), Tris-HCl, sodium azide, and sodium chloride were purchased from Fisher Scientific UK Ltd. (Leicester, UK). For determining protein content, the Bradford reagent was obtained from Biorad Laboratories (Hercules, CA). Polystyrene plates (high protein binding, flat-bottomed) were obtained from Costar® and polypropylene plates (low binding, round-bottomed) from Sigma-Aldrich (Poole, UK).

The equipment used included a plate washer (MRW, 8-channel plate washer made by

Dynex Technologies) and a plate reader (MRX microplate reader, The Microtiter® Company), two peristaltic pumps (Watson Marlow, Falmouth, UK), AKTA FPLC (Amersham Biosciences), and a vacuum concentrator (Thermo Savant, Holbrook, NY).

Fish estrogenization

Mixed-sex, sexually mature ninespine stickleback were collected from streams in Lincolnshire, UK (53°25′18.72″N, 0°10′27.48″E) in April 2013. The fish were brought to the Cefas Weymouth Laboratory where they were held in two 150-litre tanks (holding about 200 fish each) for 10 days before the treatment commenced. During this acclimation period, the temperature was raised from 12 to 15°C and the photoperiod was reduced from a light/dark cycle of 18:6 hours to 12:12 hours. Fish were fed red mosquito larvae daily. Fish remained at these conditions until the end of the treatment, which involved waterborne exposure to EE_2 at 100 $ng \cdot L^{-1}$ (±10%) over a 21-day period using a flow-through system. This concentration of EE_2 is particularly high (i.e. not environmentally relevant) and as such is effective in inducing VTG production in the liver in amounts suitable for isolation and purification, as demonstrated in a number of fish species (e.g. Scholz *et al.*, 2004). The protein yield for this purpose is higher in male plasma because male fish lack an ovary where VTG is normally deposited after hepatic production and blood circulation.

At the end of the exposure period, blood was collected from the severed caudal peduncle of estrogenized male and female fish under terminal anaesthesia (MS222) using heparinized haematocrit tubes (100 μL capacity) under license PPL 30/2117. Blood was transferred to 0.5-mL micro-centrifuge tubes and stored briefly on ice prior to being centrifuged at 10,000 *g* for 10 minutes at 4°C. After centrifugation, plasma was pooled, snap frozen in liquid nitrogen, and stored at −80°C prior to purification of VTG. Plasma from an additional group of unexposed male fish (as verified post blood collection by visual inspection of gonads) was also sampled and stored at −80°C as a baseline for size-exclusion elution profiles.

Purification of vitellogenin for antiserum production

Batches of pooled plasma (~90 μL) were defrosted and mixed 1:2 (v/v) with 0.05 M Tris-HCl elution buffer (50 mM Tris-HCl, 0.9% w/v NaCl, 0.01% w/v sodium azide, pH 7.6) containing anti-protease cocktail. The buffer was previously filtered through a VacuCap® 60 filter unit, with a 0.2-μm Supor® membrane (Pall, Gelman laboratory). Diluted plasma was then centrifuged for 5 minutes at 10,000 *g* at 4°C and passed through a 0.2-μm VectaSpin filter unit with Anopore membrane (Whatman) prior to column loading. In the meantime, a Superdex 200HR size-exclusion chromatography column (GE Healthcare) was equilibrated with the same Tris-HCl buffer used to dilute the plasma. For each run, 200 μL of filtered plasma from estrogenized fish was injected onto the column attached to an FPLC system and eluted at 0.5 $mL \cdot min^{-1}$, while absorbance (280 nm) and conductivity were monitored. Separately, plasma from untreated, immature male fish was run as above. By comparing the elution profiles between plasma of estrogenized fish and control male fish, the third peak, which demonstrated the highest absorbance, was identified as the EE_2-induced VTG protein (Fig. 3A). The 1-mL fractions containing purified VTG were collected and stored on ice slurry (~0°C) until four purification runs were completed (<4 hours). Pooled aliquots were subsequently injected onto a 1-mL HiTRAP QHP column (GE Healthcare) equilibrated

with 0.05 M Tris-HCl elution buffer and connected to the AKTA FPLC. The HiTRAP column was washed in six column volumes of elution buffer at 1 mL · min^{-1} and then a NaCl gradient up to 1 M NaCl was applied over 10 column volumes. The eluate was collected in 1-mL aliquots and the primary peak, which eluted sharply at 420 mM NaCl (Fig. 3B), was assayed for total protein concentration according to the Bradford method using bovine serum albumin as standard. The purified VTG was then divided into six aliquots, each containing 220 mg, and snap frozen in liquid nitrogen to be used for rabbit immunizations, which took place at Cambridge Biochemicals Ltd. (Cambridge, UK).

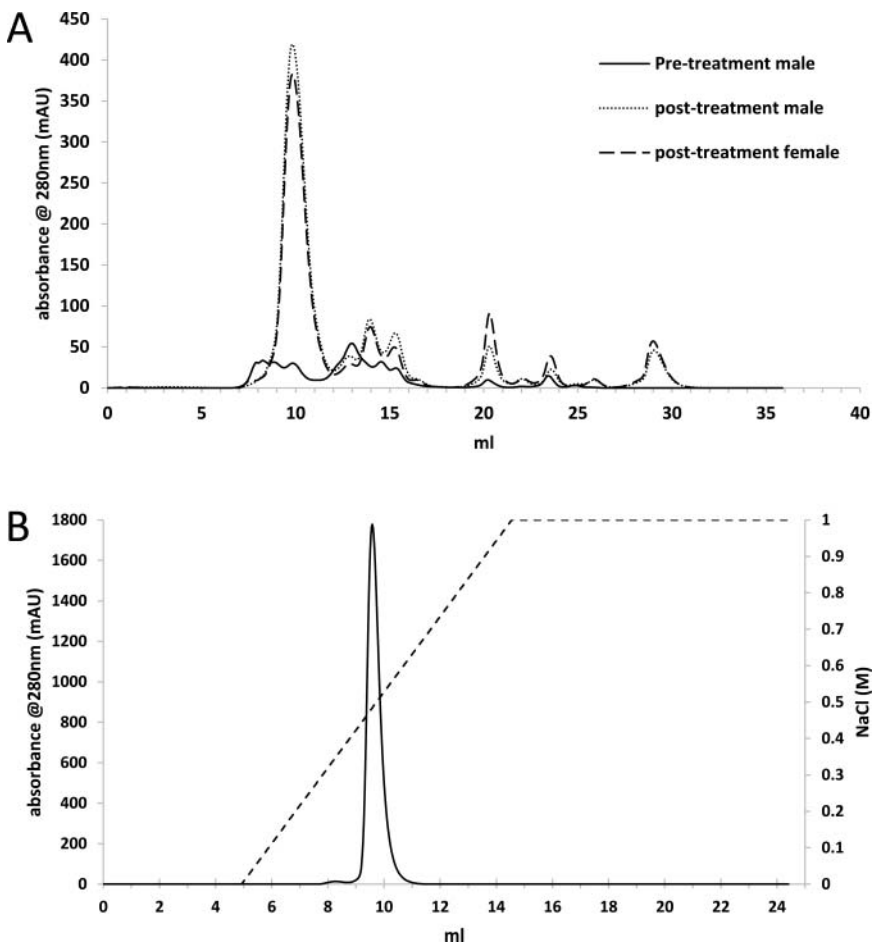


Fig. 3. (A) Size-exclusion chromatograms (Superdex 200HR) of plasma from male and female ninespine stickleback following exposure to EE_2. Elution was conducted at 0.5 mL · min^{-1} and UV was monitored at 280 nm. A chromatogram of plasma from an unexposed adult male is presented to illustrate the induction and elevation of VTG following exposure to EE_2 eluted between 9 and 11 mL. (B) Ion exchange chromatogram (HiTRAP) of fractions 9–11 mL from size-exclusion chromatography monitored at 280 nm. The primary peak around 420 mM NaCl (9–10 mL) was used for antisera production.

Purification of vitellogenin for assay standard preparation

For purifying VTG to use as the standard, the procedure was similar but the plasma was injected directly onto a HiPREP QXL 16/10 column (GE Healthcare) without prior size-exclusion chromatography. The column was washed and eluted at a flow rate of 2 mL · min^{-1} with a linear NaCl gradient (0.05–1 M) applied over 5 column volumes. Elution was monitored at 280 nm with the greatest absorbance monitored between 470 and 570 mM NaCl. A total of five 2-mL fractions were collected within this primary peak, which eluted between 65 and 75 mL. Subsamples of each 2-mL fraction, individually or combined, were subjected to SDS-PAGE (Criterion Tris-HCl 4% stacking gel, 7.5% resolving gel, pH 8.8; Biorad). Electrophoresis was carried out at 200 V (constant voltage) for 30 minutes and stained with Coomassie brilliant blue for 30 minutes following separation (Fig. 4). Sub-unit molecular mass was determined using high molecular weight markers (SDS calibration kit, Pharmacia). The SDS-PAGE profiles were compared with fractions collected earlier in the chromatogram (350–400 mM NaCl; 50–58 mL) as well as the original plasma. Based on their elution profile, the five fractions collected during the peak 470–570 mM NaCl were identified as VTG, and pooled and assayed for total protein content using the Bradford method. The purified ninespine stickleback VTG was then aliquoted into 100-μg quantities, lyophilized using a vacuum concentrator, and stored at −80°C until use. This material was used as both the quantification standard and as the coating material within the ELISA.

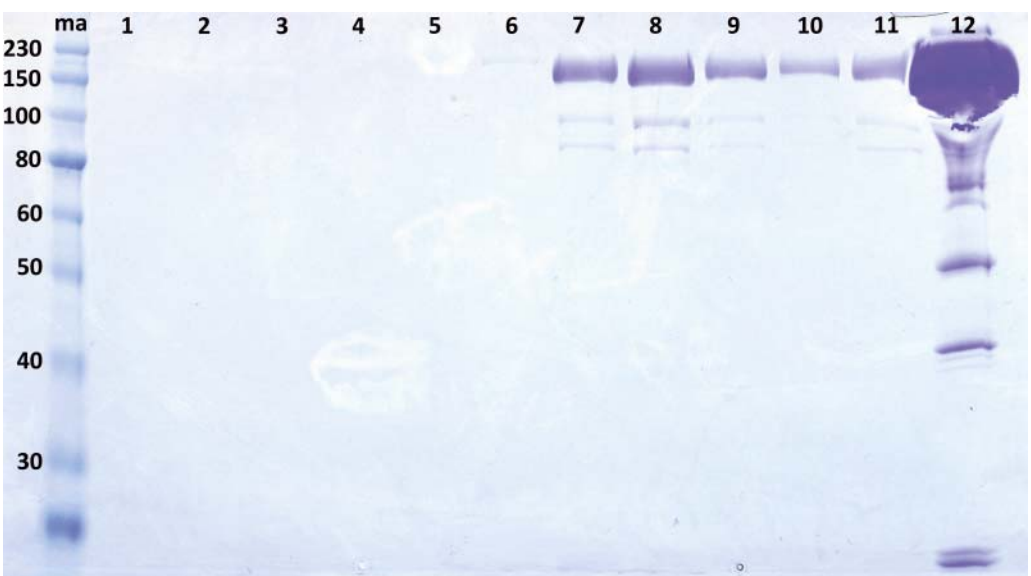


Fig. 4. SDS-PAGE of fractions from ion-exchange chromatography of pooled ninespine stickleback plasma sampled from EE_2-exposed individuals. Lanes 1–5, fractions eluted prior to VTG peak at 470 mM NaCl; lane 6, pool of lanes 1–5; lanes 7–10, fractions eluted during peak elution between 470 and 570 mM NaCl; lane 11, pool of lanes 7–10; lane 12, crude plasma diluted with elution buffer 1 : 13 (v/v). Numbers in the left margin represent size (kDa) of molecular weight markers (ma). The polypeptide fragment ~180 kDa present in lanes 7–11 represents purified VTG.

ELISA development

Several experiments were conducted to optimize the ELISA procedure with respect to the amount of coating material, incubation time, and antiserum dilution. The rabbit anti-ninespine stickleback VTG was tested in various dilutions in plates coated with varying concentrations of purified VTG protein to optimize conditions of competitive ELISA (using a checkerboard plate design). A small number of samples ranging from non-estrogenized male plasma to fully induced male plasma as well as plasma from reproductively active female fish were assayed, revealing a high degree of sensitivity and specificity of the ELISA (the intra-assay variation was 6% and the inter-assay variation was 15%).

Buffer compositions used for the ELISA are as follows: *Plate coating buffer*: 0.05 M sodium bicarbonate-carbonate, pH 9.6; *VTG wash buffer*: 100 mM sodium phosphate, 140 mM sodium chloride, 27 mM potassium chloride, 0.05% Tween 20, pH 7.8; *VTG assay buffer*: same as VTG wash buffer but with 0.1% BSA and 0.15 mM sodium azide.

Standard operating procedure

The lyophilized VTG standard was dissolved in equal volumes of distilled water and plate coating buffer to give a concentration of 500 $\mu g \cdot mL^{-1}$. An aliquot of this solution (100 μL) was added to 100 mL plate coating buffer and 100 μL of this solution dispensed into each well of the high-binding, flat bottom polystyrene plates. The plates were then sealed, wrapped in moistened paper and a plastic bag, and stored at 4°C overnight. On the same day, 135 μL of assay buffer was dispensed into each well of the low-binding, round bottom polypropylene plates. The VTG standard of 0.5 $mg \cdot mL^{-1}$ was further diluted in assay buffer to give a high (SD1 = 50 $\mu g \cdot mL^{-1}$) and a low (SD2 = 10 $\mu g \cdot mL^{-1}$) standard. Each well in columns 1 and 5 and wells A to D of column 9 received 15 μL of each sample. Wells E and F of column 9 received 15 μL of the high standard, and wells G and H received 15 μL of the low standard. Using a multipipette set to 15 μL, the contents of the wells in column 1 were mixed thoroughly and 15 μL was then transferred to column 2; the procedure was repeated up to column 4. The final 15 μL was discarded and the pipette tips changed. The procedure was repeated for the samples/standards added to the wells in columns 5 and 9. The outcome was a set of four, 10-fold dilutions of all samples and standards.

Polyclonal rabbit anti-ninespine stickleback VTG serum was diluted 1 : 1,500,000 in assay buffer (1.33 μL/20 mL) and 65 μL added to each well. The plates were then sealed, wrapped in moistened paper and a plastic bag, and stored at 4°C for a second overnight incubation.

On the morning of day 2, high-binding plates were rinsed three times with 200 μL of wash buffer. A multipipette was used to transfer 150 μL from the contents of the wells of the low-binding plate into the corresponding wells of the high-binding plate. The transfer was started with column 12, and worked backwards. Pipette tips were discarded after every four columns to avoid contamination between samples. Plates were resealed and wrapped, and were incubated at room temperature for 4–6 hours. Plates were then rinsed three times with wash buffer, followed by addition of 150 μL of second antibody (anti-rabbit conjugated to phosphatase) diluted 1:15,000 in assay buffer. Plates were resealed and wrapped and incubated overnight at 4°C.

On the morning of day 3, plates were rinsed three times with distilled water, before 150 μL of p-NPP solution was added to each well. Colour development was measured

30–45 minutes after the p-NPP addition with the microplate reader at a wavelength of 405 nm. Samples are expressed as micrograms of VTG per millilitre of plasma (accounting for dilutions prior to assay) if plasma is used, or as micrograms of VTG per heart/g body weight if the blood trapped in the heart is used as a surrogate for plasma, or as micrograms of VTG/g of head and tail weight if these tissues are used for determining VTG as in the OECD TG234 (OECD, 2011). This assay presents a convenient way to identify the presence of estrogen-modulating contaminants in the Arctic.

NINESPINE STICKLEBACK HORMONE ASSAYS

Endocrine disrupting chemicals can affect not only the estrogenic system, but also any of the endocrine axes, and therefore it is important to have tools available for all common targets of endocrine disruption. Fortunately, the endocrine system in vertebrates is highly conserved, which is one of the many reasons why fish make excellent models for human and wildlife health in toxicology. For example, chemicals that are estrogenic in one species of vertebrate tend to be estrogenic across all vertebrate taxa (Sumpter and Jobling, 1995), which explains the utility of fish VTG assays. A methodological implication is that assays developed for one fish species (such as the threespine stickleback) can readily be adapted for another (such as the ninespine stickleback).

To demonstrate this, we used a thyroxine (T_4) assay that we had previously validated for the threespine stickleback (Gardell *et al.*, 2015; Petersen *et al.*, 2015) to measure T_4 concentrations in whole-body homogenates of ninespine stickleback. This commercially available ELISA kit (Total T_4, MP Biomedicals, Santa Ana, CA) was validated for use with ninespine stickleback using tests of parallelism (standard: $y = -1.820x + 2.763$, $r^2 = 0.99$; unknown: $y = -1.175x + 0.832$, $r^2 = 0.97$) and accuracy (standard addition: $r^2 = 0.97$). Samples were resuspended in enzyme immunoassay buffer (0.1 M PBS, 0.15 NaCl, 0.1% BSA, pH 7.4) and assayed in duplicate. Absorbance was measured on a plate reader at 450 nm (SpectraMax 340PC, Molecular Devices, Sunnyvale, CA). Intra- and inter-assay variability were 5.3% and 11.2%, respectively.

CONCLUSIONS

The major goals of our efforts to develop new biomarkers for the ninespine stickleback are to enhance its utility in ecotoxicology and to recruit the scientific community to develop additional resources for its application. Having the full suite of tools currently in use for the threespine stickleback also available for the ninespine stickleback would enrich the power of arctic contamination studies, especially in broad regions of the Arctic where the threespine stickleback is absent. The Arctic is experiencing unprecedented environmental change, including contamination of people and sensitive wildlife. The ninespine stickleback seems ideally suited to meet this challenge as a sentinel species because it is abundant and widespread in marine, brackish, and fresh waters throughout the Arctic, including in contaminated sites. Furthermore, we have evidence that variation in contaminant levels in ninespine stickleback mirror those in arctic indigenous human populations (Byrne *et al.*, 2015), validating its utility as a sentinel species for both ecosystem level contamination and public health.

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REFERENCES

Alaska Community Action on Toxics (ACAT). 2015. Alaska Community Action on Toxics contaminated sites database [http:www.akaction.org/].

Alaska Department of Environmental Conservation (ADEC). 2013. Site summary for Northeast Cape formerly used defense site. Alaska Department of Environmental Conservation Division of Spill Prevention and Response Contaminated Sites Program [http:dec.alaska.gov/spar/csp/sites/stlawrence.htm].

Alaska Department of Natural Resources (ADNR). 2015. Alaska resource data file [http:dnr.alaska.gov/mdfiles/alaska_resource.html; last updated 2005].

Alaska Oil and Gas Association (AOGA). 2015. Well data [http:www.aoga.org/; last updated October 2013].

Aldenhoven, J.T., Miller, M.A., Corneli, P.S. and Shapiro, M.D. 2010. Phylogeography of ninespine sticklebacks (*Pungitius pungitius*) in North America: glacial refugia and the origins of adaptive traits. *Mol. Ecol.*, **19**: 4061–4076.

Andersson, C., Katsiadaki, I., Lundstedt-Enkel, K. and Örberg, J. 2007. Effects of 17α-ethinylestradiol on EROD activity, spiggin and vitellogenin in three-spined stickleback (*Gasterosteus aculeatus*). *Aquat. Toxicol.*, **83**: 33–42.

Aoki, K., Harris, C., Katsiadaki, I. and Sumpter, J.P. 2011. Evidence suggesting that DBP has anti-androgenic effects in fish. *Environ. Toxicol. Chem.*, **30**: 1338–1345.

Ayotte, P., Dewailly, E., Bruneau, S., Careau, H. and Vézina, A. 1995. Arctic air pollution and human health: what effects should be expected? *Sci. Total Environ.*, **160/161**: 529–537.

Bard, S.M. 1999. Global transport of anthropogenic contaminants and the consequences for the arctic marine ecosystem. *Mar. Pollut. Bull.*, **38**: 356–379.

Bell, M.A. and Foster, S.A. 1994. Introduction to the evolutionary biology of the threespine stickleback. In *The Evolutionary Biology of the Threespine Stickleback* (M.A. Bell and S.A. Foster, eds.), pp. 1–27. Oxford: Oxford University Press.

Bell, M.A., Stewart, J. and Park, P.J. 2009. The world's oldest fossil threespine stickleback fish. *Copeia*, **2009**: 256–265.

Björkblom, C., Olsson, P.E., Katsiadaki, I. and Wiklund, T. 2007. Estrogen- and androgen-sensitive bioassays based on primary cell and tissue slice cultures from three-spined stickleback (*Gasterosteus aculeatus*). *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.*, **146**: 431–442.

Blais, J.M. 2005. Biogeochemistry of persistent bioaccumulative toxicants: processes affecting the transport of contaminants to remote areas. *Can. J. Fish. Aquat. Sci.*, **62**: 236–243.

Bruneaux, M., Johnston, S.E., Herczeg, G., Merilä, J., Primmer, C.R. and Vasemägi, A. 2013. Molecular evolutionary and population genomic analysis of the nine-spined stickleback using a modified restriction-site-associated DNA tag approach. *Mol. Ecol.*, **22**: 565–582.

Byrne, S., Miller, P.K., Waghiyi, V., Buck, C.L., von Hippel, F.A. and Carpenter, D.O. 2015. Persistent organochlorine pesticide exposure related to a formerly used defense site on

St. Lawrence Island, Alaska: data from sentinel fish and human sera. *J. Toxicol. Environ. Health A*, **78**: 976–992.

Dewailly, E., Nantel, A., Weber, J.-P. and Meyer, F. 1989. High levels of PCBs in breast milk of Inuit women from arctic Quebec. *Bull. Environ. Contam. Toxicol.*, **43**: 641–646.

Furin, C.G., von Hippel, F.A., Hagedorn, B. and O'Hara, T.M. 2013. Perchlorate trophic transfer increases tissue concentrations above ambient water exposure alone in a predatory fish. *J. Toxicol. Environ. Health A*, **76**: 1072–1084.

Gardell, A.M., Dillon, D.M., Smayda, L.C., von Hippel, F.A., Cresko, W.A., Postlethwait, J.H. *et al.* 2015. Perchlorate exposure does not modulate temporal variation of whole-body thyroid and androgen hormone content in threespine stickleback. *Gen. Comp. Endocrinol.*, **219**: 45–52.

Glazer, A.M., Killingbeck, E.E., Mitros, T., Rokhsar, D.S. and Miller, C.T. 2015. Genome assembly improvement and mapping convergently evolved skeletal traits in sticklebacks with genotyping-by-sequencing. *G3: Genes | Genomes | Genetics*, **5**: 1463–1472.

Grannas, A.M., Bogdal, C., Hageman, K.J., Halsall, C., Harner, T., Hung, H. *et al.* 2013. The role of the global cryosphere in the fate of organic contaminants. *Atmos. Chem. Phys.*, **13**: 3271–3305.

Guo, B., Chain, F.J.J., Bornberg-Bauer, E., Leder, E.H. and Merilä, J. 2013. Genomic divergence between nine- and three-spined sticklebacks. *BMC Genomics*, **14**: 756.

Hahlbeck, E., Katsiadaki, I., Mayer, I., Adolfsson-Erici, M., James, J. and Bengtsson, B.E. 2004. The juvenile three-spined stickleback (*Gasterosteus aculeatus* L.) as a model organism for endocrine disruption II. Kidney hypertrophy, vitellogenin and spiggin induction. *Aquat. Toxicol.*, **70**: 311–326.

Jolly, C., Katsiadaki, I., Le Belle, N., Mayer, I. and Dufour, S. 2006. Development of a stickleback kidney cell culture assay for the screening of androgenic and anti-androgenic endocrine disrupters. *Aquat. Toxicol.*, **79**: 158–166.

Jolly, C., Katsiadaki, I., Morris, S., Le Belle, N., Dufour, S., Mayer, I. *et al.* 2009. Detection of the anti-androgenic effect of endocrine disrupting environmental contaminants using *in vivo* and *in vitro* assays in the three-spined stickleback. *Aquat. Toxicol.*, **92**: 228–239.

Jones, F.C., Grabherr, M.G., Chan, Y.F., Russell, P., Mauceli, E., Johnson, J. *et al.* 2012. The genomic basis of adaptive evolution in threespine sticklebacks. *Nature*, **484**: 55–61.

Katsiadaki, I., Scott, A.P., Hurst, M.R., Matthiessen, P. and Mayer, I. 2002a. Detection of environmental androgens: a novel method based on enzyme-linked immunosorbent assay of spiggin, the stickleback (*Gasterosteus aculeatus*) glue protein. *Environ. Toxicol. Chem.*, **21**: 1946–1954.

Katsiadaki, I., Scott, A.P. and Mayer, I. 2002b. The potential of the three-spined stickleback, *Gasterosteus aculeatus* L., as a combined biomarker for oestrogens and androgens in European waters. *Mar. Environ. Res.*, **54**: 725–728.

Katsiadaki, I., Morris, S., Squires, C., Hurst, M.R., James, J.D. and Scott, A.P. 2006. Use of the three-spined stickleback (*Gasterosteus aculeatus*) as a sensitive *in vivo* test for detection of environmental antiandrogens. *Environ. Health Perspect.*, **114 (**suppl. 1): 115–121.

Katsiadaki, I., Sanders, M., Sebire, M., Nagae, M., Soyano, K. and Scott, A.P. 2007. Three-spined stickleback: an emerging model in environmental endocrine disruption. *Environ. Sci.*, **14**: 263–283.

Katsiadaki, I., Williams, T.D., Ball, J.S., Bean, T.P., Sanders, M.B., Wu, H. *et al.* 2010. Hepatic transcriptomic and metabolomic responses in the stickleback (*Gasterosteus aculeatus*) exposed to ethinyl-estradiol. *Aquat. Toxicol.*, **97**: 174–187.

Katsiadaki, I., Sanders, M.B., Henrys, P.A., Scott, A.P., Matthiessen, P. and Pottinger, T.G. 2012. Field surveys reveal the presence of anti-androgens in an effluent-receiving river using stickleback-specific biomarkers. *Aquat. Toxicol.*, **122/123**: 75–85.

Kawahara, R., Miya, M., Mabuchi, K., Near, T.J. and Nishida, M. 2009. Stickleback phylogenies resolved: evidence from mitochondrial genomes and 11 nuclear genes. *Mol. Phylogenet. Evol.*, **50**: 401–404.

Keivany, Y. and Nelson, J.S. 2000. Taxonomic review of the genus *Pungitius*, ninespine sticklebacks (Gasterosteidae). *Cybium*, **24**: 107–122.

Kenney, L.A. and von Hippel, F.A. 2014. Morphological asymmetry of insular freshwater populations of threespine stickleback. *Environ. Biol. Fish.*, **97**: 225–232.

Kenney, L.A., von Hippel, F.A., Willacker, J.J. and O'Hara, T.M. 2012. Mercury concentrations of a resident freshwater forage fish at Adak Island, Aleutian Archipelago, Alaska. *Environ. Toxicol. Chem.*, **31**: 2647–2652.

Kenney, L.A., Eagles-Smith, C.A., Ackerman, J.T. and von Hippel, F.A. 2014. Temporal variation in fish mercury concentrations within lakes from the western Aleutian Archipelago, Alaska. *PLoS One*, **9**: e102244.

Luoma, S.N. and Rainbow, P.S. 2008. *Metal Contamination in Aquatic Environments: Science and Lateral Management*. Cambridge: Cambridge University Press.

Ma, J., Hung, H., Tian, C. and Kallenborn, R. 2011. Revolatilization of persistent organic pollutants in the Arctic induced by climate change. *Nature Climate Change*, **1**: 255–260.

Mattern, M.Y. 2007. Phylogeny, systematics and taxonomy of sticklebacks. In *Biology of the Three-spined Stickleback* (S. Östlund-Nilsson, I. Mayer and F.A. Huntingford, eds.), pp. 1–40. Boca Raton, FL: CRC Press.

McKinney, M.A., Pedro, S., Dietz, R., Sonne, C., Fisk, A.T., Roy, D. *et al.* 2015. A review of ecological impacts of global climate change on persistent organic pollutant and mercury pathways and exposures in arctic marine ecosystems. *Curr. Zool.*, **61**: 617–628.

Merilä, J. 2013. Nine-spined stickleback (*Pungitius pungitius*): an emerging model for evolutionary biology research. *Ann. NY Acad. Sci.*, **1289**: 18–35.

Miller, M.R., Dunham, J.P., Amores, A., Cresko, W.A. and Johnson, E.A. 2007. Rapid and cost-effective polymorphism identification and genotyping using restriction site associated DNA (RAD) markers. *Genome Res.*, **17**: 240–248.

Nagae, M., Kawasaki, F., Tanaka, Y., Ohkubo, N., Matsubara, T., Soyano, K. *et al.* 2007. Detection and assessment of androgenic potency of endocrine-disrupting chemicals using three-spined stickleback, *Gasterosteus aculeatus*. *Environ. Sci.*, **14**: 255–261.

Norris, D.O. and Carr, J.A. 2006. *Endocrine Disruption*. Oxford: Oxford University Press.

Noyes, P.D. and Lena, S.C. 2015. Forecasting the impacts of chemical pollution and climate change interactions on the health of wildlife. *Curr. Zool.*, **61**: 669–689.

Organization for Economic Cooperation and Development (OECD). 2011. OECD Guideline for the Testing of Chemicals, Section 2: Effects on Biotic Systems. Test No. 234: Fish Sexual Development Test [http:www.oecd-ilibrary.org/environment/test-no-234-fish-sexual-development-test_9789264122369-en].

Organization for Economic Cooperation and Development (OECD). 2012. Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption, Series on Testing Assessment No. 150. Paris: OECD ENV/JM/MONO(2012)22 [http:www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote = env/jm/mono(2012)22&doclanguage = en].

Ozsolak, F. and Milos, P.M. 2011. RNA sequencing: advances, challenges and opportunities. *Nature Rev. Genet.*, **12**: 87–98.

Petersen, A.M., Dillon, D., Bernhardt, R.A., Torunsky, R., Postlethwait, J.H., von Hippel, F.A. *et al.* 2015. Perchlorate disrupts embryonic androgen synthesis and reproductive development in threespine stickleback without changing whole-body levels of thyroid hormone. *Gen. Comp. Endocrinol.*, **210**: 130–144.

Piña, B., Casado, M. and Quirós, L. 2007. Analysis of gene expression as a new tool in ecotoxicology and environmental monitoring. *Trends Anal. Chem.*, **26**: 1145–1154.

Pottinger, T.G., Carrick, T.R. and Yeomans, W.E. 2002. The three-spined stickleback as an environmental sentinel: effects of stressors on whole-body physiological indices. *J. Fish Biol.*, **61**: 207–229.

Pottinger, T.G., Cook, A., Jürgens, M.D., Rhodes, G., Katsiadaki, I., Balaam, J.L. *et al.* 2011a.

Effects of sewage effluent remediation on body size, somatic RNA:DNA ratio, and markers of chemical exposure in three-spined sticklebacks. *Environ. Int.*, **37**: 158–169.

Pottinger, T.G., Cook, A., Jürgens, M.D., Sebire, M., Henrys, P.A., Katsiadaki, I. *et al.* 2011b. Indices of stress in two populations of three-spined sticklebacks are altered by extreme weather events and exposure to waste-water effluent. *J. Fish Biol.*, **79**: 256–279.

Pottinger, T.G., Katsiadaki, I., Jolly, C., Sanders, M., Mayer, I., Scott, A.P. *et al.* 2013. Anti-androgens act jointly in suppressing spiggin concentrations in androgen-primed female three-spined sticklebacks: prediction of combined effects by concentration addition. *Aquat. Toxicol.*, **140/141**: 145–156.

Rastas, P., Calboli, F.C.F., Guo, B., Shikano, T. and Merilä, J. 2016. Construction of ultradense linkage maps with Lep-MAP2: stickleback F_2 recombinant crosses as an example. *Genome Biol. Evol.*, **8**: 78–93.

RdB. 2015. Robin des Bois, Polar star: 2750 contaminated sites in Arctic [http:www.robin desbois.org/arctic/polar_star_2_EN.html; last updated December 2009; accessed October 2015].

Sanchez, W., Katsiadaki, I., Piccini, B., Ditche, J.M. and Porcher, J.M. 2008. Biomarker responses in wild three-spined stickleback (*Gasterosteus aculeatus* L.) as a useful tool for freshwater biomonitoring: a multiparametric approach. *Environ. Int.*, **34**: 490–498.

Sanders, M., Sebire, M., Sturve, J., Christian, P., Katsiadaki, I., Lyons, B.P. *et al.* 2008. Exposure of sticklebacks (*Gasterosteus aculeatus*) to cadmium sulfide nanoparticles: biological effects and the importance of experimental design. *Mar. Environ. Res.*, **66**: 161–163.

Santos, E.M., Ball, J.S., Williams, T., Wu, H., Ortega, F., van Aerle, R. *et al.* 2010. Identifying health impacts of exposure to copper using transcriptomics and metabolomics in a fish model. *Environ. Sci. Technol.*, **44**: 820–826.

Scholz, S., Kordes, C., Hamann, J. and Gutzeit, H.O. 2004. Induction of vitellogenin *in vivo* and *in vitro* in the model teleost medaka (*Oryzias latipes*): comparison of gene expression and protein levels. *Mar. Environ. Res.*, **57**: 235–244.

Sebire, M., Allen, Y., Bersuder, P. and Katsiadaki, I. 2008. The model antiandrogen flutamide suppresses the expression of typical male stickleback reproductive behaviour. *Aquat. Toxicol.*, **90**: 37–47.

Sebire, M., Scott, A.P., Tyler, C.R., Cresswell, J., Hodgson, D.J., Morris, S. *et al.* 2009. The organo-phosphorous pesticide, fenitrothion, acts as an anti-androgen and alters reproductive behavior of the male three-spined stickleback, *Gasterosteus aculeatus*. *Ecotoxicology*, **18**: 122–133.

Sebire, M., Katsiadaki, I., Taylor, N.G.H., Maack, G. and Tyler, C.R. 2011. Short-term exposure to a treated sewage effluent alters reproductive behaviour in the three-spined stickleback (*Gasterosteus aculeatus*). *Aquat. Toxicol.*, **105**: 78–88.

Serreze, M.C. and Barry, R.G. 2011. Processes and impacts of arctic amplification: a research synthesis. *Global Planet. Change*, **77**: 85–96.

Shapiro, M.D., Summers, B.R., Balabhadra, S., Aldenhoven, J.T., Miller, A.L., Cunningham, C.B. *et al.* 2009. The genetic architecture of skeletal convergence and sex determination in ninespine sticklebacks. *Curr. Biol.*, **19**: 1140–1145.

Shikano, T., Ramadevi, J., Shimada, Y. and Merilä, J. 2010. Utility of sequenced genomes for microsatellite marker development in non-model organisms: a case study of functionally important genes in nine-spined sticklebacks (*Pungitius pungitius*). *BMC Genomics*, **11**: 334.

Shikano, T., Natri, H.M., Shimada, Y. and Merilä, J. 2011. High degree of sex chromosome differentiation in stickleback fishes. *BMC Genomics*, **12**: 474.

Shikano, T., Laine, V.N., Herczeg, G., Vilkki, J. and Merilä, J. 2013. Genetic architecture of parallel pelvic reduction in ninespine sticklebacks. *G3: Genes | Genomes | Genetics*, **3**: 1833–1842.

Snell, T.W., Brogdon, S.E. and Morgan, M.B. 2003. Gene expression profiling in ecotoxicology. *Ecotoxicology*, **12**: 475–483.

Sumpter, J.P. and Jobling, S. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ. Health Perspect.*, **103**: 173–178.

Tierney, K.B., Farrell, A.P. and Brauner, C.J. 2014. *Organic Chemical Toxicology of Fishes*. Amsterdam: Academic Press.

von Hippel, F.A. 2010. Physiology and behavior. In *Tinbergen's Legacy in Behaviour: Sixty Years of Landmark Stickleback Papers* (F.A. von Hippel, ed.). Leiden: Brill Academic Publishers.

Wang, C., Shikano, T., Persat, H. and Merilä, J. 2015. Mitochondrial phylogeography and cryptic divergence in the stickleback genus *Pungitius*. *J. Biogeogr.*, **42**: 2334–2348.

Wania, F. and Mackay, D. 1993. Global fractionation and cold condensation of low volatility organochlorine compounds in polar regions. *Ambio*, **22**: 10–18.

Wania, F. and Mackay, D. 1996. Tracking the distribution of persistent organic pollutants. *Environ. Sci. Technol.*, **30**: 390–396.

Williams, T., Wu, H., Santos, E.M., Ball, J.S., Katsiadaki, I., Brown, M.M. *et al.* 2009. Hepatic transcriptomic and metabolomic responses in the stickleback (*Gasterosteus aculeatus*) exposed to environmentally relevant concentrations of dibenzanthracene. *Environ. Sci. Technol.*, **43**: 6341–6348.

Wöhler, F. 1828. Ueber künstliche Bildung des Harstoffs. *Annalen der Physik und Chemie*, **88**: 253–256.