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Lake charr (*Salvelinus namaycush*) clotting response may act as a plasma biomarker of sea lamprey (*Petromyzon marinus*) parasitism: Implications for management and wound assessment

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ABSTRACT

Sea lampreys (*Petromyzon marinus*) have decimated the Great Lakes fisheries over the past century, and their control is central to protecting native fish populations. Wounding data collected from host fish, including classifying wounds as Type A (penetrating the integument) or Type B (superficial), is an integral part of sea lamprey monitoring efforts and helps inform management decisions; however, wound assessment is subjective and error-prone. This study aimed to determine if protein biomarkers of parasitism could be quantified in host fish plasma to serve as a potential objective aide in current wound assessment practices. Male siscowet lake charr (*Salvelinus namaycush*) were parasitized in a lab setting for four days, after which the sea lamprey was removed, host blood was collected, and the wound Type recorded. A second blood sample was collected from host fish with Type A wounds 7 months later. The plasma proteome was quantified using iTRAQ, and the relative abundances of 169 proteins were compared between parasitized and non-parasitized control fish. Three functional classes of proteins were modified by sea lamprey parasitism: immune response, lipid transport, and blood coagulation. A major finding was evidence of a concerted anticoagulation response in fish with Type A wounds, including changes in protein components and regulators of fibrin clot formation, some of which did not fully recover within 7 months. A modified clotting assay yielded a smaller thrombin-induced fibrin clot from parasitized fish, supporting the proteomic results. Therefore, measuring blood clottability could improve sea lamprey damage estimates by providing a more objective and quantitative index of parasitism than is offered by wounding data alone.

Introduction

The landlocked sea lamprey (*Petromyzan marinus*) is an ectoparasitic cyclostome that feeds on the blood and tissue of large-bodied teleost fish, including its preferred host, lake charr (*Salvelinus namaycush*). Lake charr harvest from the Laurentian Great Lakes plummeted from ~6 million kilograms in 1944 to less than 0.1 million kilograms in 1964 (Baldwin et al., 2018). Sea lamprey predation leading to host mortality is considered a major contributing factor in this historic collapse of the commercial fishery (Ebener et al., 2003; Pycha, 1980), along with over-fishing and habitat degradation (Hansen, 1999). The presence of sea lamprey in the Great Lakes is an ongoing challenge that costs the Canadian and United States governments millions of dollars in sea lamprey monitoring and control measures every year (Fetterolf Jr., 1980; Koonce et al., 1993; Talhelm and Bishop, 1980). Effective control of sea lamprey population size is critical to stabilizing the Great Lakes ecosystem.

Sea lamprey control measures in the Great Lakes involve a multi-pronged approach that exploits the complex life cycle of these fish to target accessible and vulnerable life stages. Larval sea lampreys (ammocetes) are found buried in streambeds throughout the Great Lakes basin, where they spend several years filter feeding from the water column (Beamish, 1980). The accessibility and immobility of this life stage makes it amenable

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to chemical control using the lampricides 3-trifluoromethyl-4-nitrophenol (TFM) and niclosamide, and targeted lampricide treatment of nursery streams is a highly effective and crucial component of sea lamprey control (Wilkie et al., 2019). Ammocetes that survive through the larval stage metamorphose into parasitic juveniles, complete with a toothed oral disc and rasping tongue that are used for attaching to and feeding from host fish once they migrate downstream to open waters (Beamish, 1980). After 12–20 months as juvenile parasites, sea lampreys migrate to nursery sites as sexually mature adults where they spawn and die. Efforts to minimize sea lamprey spawning include barriers that block the upstream migration, coordinated trapping and culling initiatives, application of lamprey-specific pheromones to reorient spawners, and the release of sterile males (Christie and Goddard, 2003; Smith and Tibbles, 1980). This integrative pest management system has successfully reduced the sea lamprey population in the Great Lakes and remains essential to continued population control (Christie and Goddard, 2003; Smith and Tibbles, 1980; Wilkie et al., 2019). The cost and effort involved in resource deployment necessitates prioritizing control measures to select sites within the Great Lakes basin; therefore, the Great Lakes Fishery Commission oversees a sea lamprey assessment program to estimate lamprey-induced mortality of host fish (Bence et al., 2003; Schneider et al., 1996; Sitar et al., 1999), gauge the success of sea lamprey control efforts (Adams et al., 2003; Rutter and Bence, 2003), and justify the allocation of resources directed at sea lamprey control (Irwin et al., 2012).

Sea lamprey parasites leave a visual record of attack on host fish. During feeding, the rasping tongue grates at the host integument to open a wound and initiate blood flow, while secretions from the paired buccal glands of the parasite, known as lamphredin, facilitate continued feeding through its anticoagulant, anesthetic, and other properties (Lennon, 1954; Xiao et al., 2012). Duration and intensity of the parasite attack varies such that some host fish may survive with little evidence of the encounter, while other host fish succumb to death from blood loss and infection (Farmer, 1980; Farmer et al., 1975). Tallying and classifying the parasite-inflicted wounds and resulting scars (collectively called 'marks') on host fish helps estimate the abundance of sea lamprey parasites and their associated damage in the Great Lakes, and is considered a valuable metric in sea lamprey assessment (Bence et al., 2003; Irwin et al., 2012; Lantry et al., 2015). According to the King Jr. (1980) classification system, sea lamprey marks are categorized by severity, where Type A indicates a severe wound that penetrated to the underlying muscle, and Type B indicates a mild wound that failed to break the integument (King Jr., 1980). Marks are further scored into four stages of healing, ranging from a freshly inflicted wound (I) to a fully healed scar (IV) (King Jr., 1980). Though valuable, wound assessment is inherently observational and subjective, and despite standardized classification procedures there remains a large amount of error associated with the correct identification of wound Type and healing stage (Ebener et al., 2003; Firkus et al., 2020). For example, a recent workshop found that a group of experts in the field were only able to correctly identify wound Type 47% of the time, while both wound Type and healing stage were correctly identified only 29% of the time (Firkus et al., 2020). Moreover, the reliance on recent and severe marks (AI-AIII) for estimating the population size of juvenile sea lamprey and their threat to host populations (Lantry et al., 2015; Rutter and Bence, 2003) does not account for host fish with unanticipated healing trajectories (Nowicki, 2008) and mortality in hosts with Type B wounds (Firkus et al., 2020). Therefore, quantitative biomarkers in host fish that define the severity, dynamics or latency of a sea lamprey predation event could help to validate mark classifications and make wounding data a more reliable metric for sea lamprey assessment.

Blood biomarkers are commonly used in human and veterinary medicine for health assessment and as diagnostic tools. Blood is the ideal tissue for such tools because it circulates through all organs and represents a collective pool of constituent and secreted proteins. Moreover, blood collection is simple, minimally invasive, and non-lethal. Collection of blood samples from fish in the field is a relatively simple procedure that involves training personnel to target the caudal vein, and requires minimal equipment (needles, storage vials, ice). Plasma can be separated using field-ready portable centrifuges and then stored in regular freezers. If blood biomarkers of sea lamprey parasitism are present in host fish, it could be feasible to incorporate blood collection into lake charr population monitoring programs and sea lamprey marking data collection. By augmenting existing monitoring efforts with biomarker quantification, a more accurate and complete picture of sea lamprey populations and their estimated impact on lake charr may be achieved, ultimately providing stronger justification for the implementation of costly sea lamprey control measures. Furthermore, if blood biomarkers can be identified that are more precise and less prone to error than traditional wound classification, fewer fish would need to be sampled during lake charr monitoring efforts to accurately characterize sea lamprey damage. As a first step towards this goal, the objective of this study was to determine if protein biomarkers of sea lamprey parasitism can be identified in the plasma of parasitized lake charr, and, if so, whether the abundances of these biomarkers vary with attack intensity (wound Type) and recovery time.

Methods

Fish procurement and husbandry

Thirteen sexually mature male siscowet lake charr (630–786 mm, 1.86–4.14 kg) reared in a laboratory setting were used for this study, and were previously implanted with PIT-tags for individual identification. Siscowets are a deep-water lake charr ecomorph found in Lake Superior and are frequently used in studies assessing the effects of sea lamprey parasitism (Goetz et al., 2016; Smith et al., 2016). The fish were hatched from embryos collected from wild Lake Superior siscowets in 2006, and then raised in 1.5 × 21 m raceways located in an unheated outbuilding under ambient light and temperature at the University of Wisconsin-Stevens Point (UWSP) Northern Aquaculture Demonstration Facility (NADF; Bayfield, WI, USA). Raceways were supplied with onsite well water (~250 L/min). The fish were fed a maintenance diet (0.5%) of Rangen 8.0 mm EXTR 450 Trout Feed (Buhl, ID, USA), and excess uneaten food was observed at each feeding. Fish were not fed during the parasitism trials. Actively parasitic sea lampreys (47–204 g) were collected from wild lake charr hosts in Lake Superior and Lake Huron by commercial fishing operations during the summer and early-autumn of 2017. Sea lampreys were screened for disease prior to use and were held in separate flow-through tanks at UWSP NADF while not in use for parasitism trials. All protocols in this study were approved by the UWSP and Michigan State University Animal Care and Use Committees.

Experimental design and sampling

Parasitism trials took place in November and December of 2017 when sea lamprey parasitism is seasonally peaking in the wild (Bergstedt and Swink, 1995; Lantry et al., 2015). Lake charr were placed in individual 1000 L circular tanks (water temperature 6.8–8.3 °C; dissolved oxygen 9.9–12.2 mg/L) with a single sea lamprey (n = 9) or without a sea lamprey (non-parasitized control, NP; n = 4). Sea lampreys were allowed to feed naturally for 4 days from

the time of attachment, at which point the host fish was anesthetized in 60 mg/L buffered tricaine methane sulfonate (MS-222), and the sea lamprey parasites were manually removed. The wounds were examined and classified as either Type A (A; n = 4) or Type B (B; n = 5) following the current classification guidelines (Ebener et al., 2006). A 0.5 ml blood sample was collected from the caudal vein with a 23 G needle and syringe coated with 1000 IU/ml heparin, and then centrifuged at 1500 *g* for 10 min at ambient temperature. The plasma was separated and stored at −80 °C. Lake charr were placed back in the raceways and allowed to recover. Following 7 months recovery (July 2018), a second blood sample was collected as above. Due to technical constraints on sample sizes, recovery samples from the non-parasitized control fish and fish with Type A wounds were prioritized over fish with Type B wounds for the proteomic analysis.

Protein isolation and iTRAQ labeling

Lake charr plasma samples (n = 21) were prepared for trypsin digestion and iTRAQ labelling as previously described (Alderman et al., 2017; Dindia et al., 2017). Briefly, samples were thawed on ice, vortexed, and centrifuged at 15,000 *g* for 15 min at 4 °C. A 5 µl aliquot from each sample was pooled to serve as an internal control for plex standardization. Plasma was diluted 10-fold in SDS buffer (4% w/v sodium dodecyl sulphate, 100 mM HEPES, 0.1 M dithiothreitol, pH 7.6) containing 1x protease inhibitor (Roche, Mississauga, ON), incubated at room temperature for 30 min, and then precipitated overnight (Calbiochem Protein Precipitation Kit, EMD Millipore, Billerica, MA). The resulting protein pellet was reconstituted in HEPES buffer (1 M HEPES, 8 M urea, 2 M thiourea, 4% CHAPS w/v; pH 8.5) and total protein quantified using the Pierce 660 Protein Assay (Thermo-Fisher Scientific, Ottawa, ON) with bovine serum albumin standards. For each sample, including triplicates of the pooled internal control, 100 µg of total protein was transferred to a pre-equilibrated 10 kDa Amicon centrifugation filter (EMD Millipore), washed 3 times with UA buffer (8 M urea in 0.1 M HEPES, pH 8.5), and then treated with 0.05 M iodoacetamide (Sigma-Aldrich, Oakville, ON) for 30 min in the dark. Sample buffer was then exchanged to 0.5 M triethylammonium bicarbonate (Sigma-Aldrich) with 3 sequential washes, and proteins were digested overnight at 37 °C using sequence-modified trypsin (Promega Corporation, Madison, WI) at a 1:50 enzyme:protein ratio. Digested peptides were recovered and then labeled for 2 h at room temperature using isobaric tags from three 8-plex iTRAQ kits (SCIEX, Framingham, MA) following the manufacturer's protocol. Labels were evenly distributed across experimental groups such that each of the 3 plexes contained an internal control sample, a representative sample from all experimental conditions (NP, A, B), and paired time samples for individual fish (x = immediately after parasitism; y = 7-month recovery). After labeling, samples were pooled by plex, desalted by solid phase extraction through a C18 column (Sigma-Aldrich), and eluted with 70% acetonitrile (ACN) and 0.1% formic acid.

Mass spectrometry, bioinformatics, and differential abundance

Samples were analyzed by LC-MS/MS at the core mass spectrometry lab of Bioinformatics Solutions Incorporated (BSI; Waterloo, ON, Canada). Briefly, each sample was analyzed by nanoflow liquid chromatography on an Ultimate 3000 LC system online-coupled to a Fusion Lumos Tribrid mass spectrometer through a nanoelectrospray flex-ion source (ThermoFisher Scientific). Samples were loaded onto a 5 mm µ-precolumn (ThermoFisher Scientific) with 300 µm inner diameter filled with 5 µm C18 PepMap100 beads, and then separated using a 15 cm × 75 µm PepSEP column. Peptides were eluted (150 min at 250 nL/min) using a linear gradient from 4 to 32% ACN in 0.1% formic acid and introduced into the mass spectrometer by electrospray ionization. The linear gradient was followed by a 95% ACN phase and a 4% ACN phase to clean and equilibrate the column. The Fusion Lumos was operated in data-dependent mode, switching automatically between one fill scan and subsequent MS/MS scans of the most abundant peaks with a cycle time of 3 sec. Data was acquired using an FT-IT method to take advantage of the speed of the instrument. MS1 acquisition was performed with a scan range of 400–1600 *m*/*z* with resolution set to 120 000, automatic maximum injection AGC target set to 1×10^5. Isolation for MS2 scans was performed in the quadrupole, with an isolation window of 0.7 Da. MS2 scans were done in the Orbitrap with an automatic maximum injection AGC target of 1×10^5 and resolution of 45 000. Ions were selected for HCD fragmentation with a normalized collision energy of 36. The first mass was fixed at 110 *m*/*z*. Dynamic exclusion was applied using a maximum exclusion list of 500 with an exclusion duration of 70 sec and a mass tolerance of ± 10 ppm.

LC-MS/MS data were searched in PEAKS Studio X Pro (BSI) using a parent tolerance of 10 ppm and a fragment tolerance of 0.02 Da. In total, 391 285 entries were searched against a database containing sequences of all lower taxa of *Salmonidae* downloaded from the Uniprot database (December 7, 2020). Fixed modifications included carbidomethylation (+57.02 Da on cysteine), and iTRAQ 8plex (+304.20 Da on lysine and peptide N-termini), and variable modifications included oxidation (+15.99 Da on methionine) and deamidation (+0.98 Da on asparagine and arginine). Proteins that passed the false discovery rate threshold (FDR; 1% at the peptide and protein levels) were quantified in PEAKS Studio X Pro using the iTRAQ reporter ions, and data exported. Protein identifications assigned as 'uncharacterized proteins' from the *Salmonidae* search were manually blasted within the Uniprot database and re-assigned if a homologous protein (>80% identity) was found.

The full list of identified proteins from all three plexes was collated in RStudio and filtered for data quality so that only proteins identified on all three plexes and by more than one unique peptide were used for statistical analysis. Protein abundances were scaled to the internal control sample across the 3 plexes, and then differential protein expression was carried out using the Bioconductor package DEP (Zhang et al., 2018) with pairwise comparisons to determine the immediate effects of parasitism (NPx vs Bx, NPx vs Ax, Bx vs Ax) and the extent of recovery (NPy vs Ay, Ax vs Ay). A double criteria threshold approach was applied to identify proteins of interest (Alderman et al., 2020, 2019), such that only proteins with both a p-value < 0.05 and an absolute log2 fold change (FC) > 0.263 were considered differentially abundant (DA). Protein expression data is plotted as log2 abundance to facilitate FC visualizations from the subtraction of means.

Modified clottability assay

The observed changes in proteins related to blood coagulation suggest a reduced capacity for host fish to form a fibrin clot. Given sample volume limitations and that heparin was used as an anticoagulant, a modified clottability assay (Mackie et al., 2003) was used to cross-validate the proteomics results, using the same plasma samples from the iTRAQ experiment as well as plasma collected from additional non-parasitized and Type A wound fish (n = 5 per treatment). A 200 µl aliquot of each plasma sample was equilibrated to 37 °C in a microcentrifuge tube pre-weighed using a high precision balance, and then clotting was initiated with the addition of an excess of Pacific Hemostasis Bovine Thrombin (1 NIH U/ml; Thermo-Fisher). Samples were incubated for 30 min at 37 °C, briefly centrifuged (1000 *g* for 1 min), and any unclotted plasma carefully removed with a pipet. The fibrin clot was rinsed once with 200 µl milliQ water and dried overnight at 37 °C to con-

stant weight, and then the tube was re-weighed to calculate dry clot weight. It was assumed that any influence of the trace amounts of heparin on fibrin clot formation would be similar across experimental samples and further minimized through the addition of excess thrombin and an extended incubation time. Differences in dry clot weight between treatments were determined using a Kruskal-Wallis test. To demonstrate that dry clot weight can be used as an indirect estimate of initial plasma fibrinogen concentration, Pacific Hemostasis Reference Plasma (ThermoFisher; manufacturer specified fibrinogen concentration = 304 m g/dL) was serially diluted and used in the modified clottability assay described above.

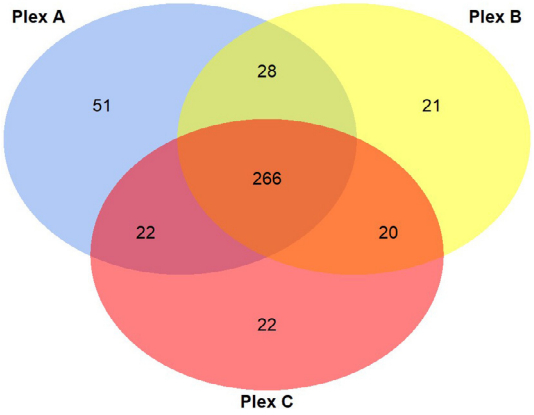

Fig. 1. Venn diagram comparing the numbers of common proteins between the three iTRAQ plexes from a total of 430 unique protein identifications. The 266 unique protein identities that were common to all plexes were pared for quality control prior to differential expression analysis.

Results

Characterizing the plasma proteome of lake charr

A combined total of 430 unique proteins were quantified across the three iTRAQ plexes, with 266 proteins common to all (Fig. 1). The list of common proteins was pared through a series of quality assurance steps to remove replicated entries and proteins identified by a single unique peptide (91 proteins), proteins with extreme oppositional abundances between the internal control samples (5 proteins), and entries with missing values (1 protein). The resulting lake charr plasma proteome was characterized by 169 high confidence protein identifications and quantifications that spanned 3 orders of magnitude (Electronic Supplementary Material Table S1). The ranked mean abundances of proteins from non-parasitized fish showed that the male siscowet lake charr plasma proteome is largely comprised of transport proteins (ex. serotransferrin, hemopexin, apolipoproteins), fibrinogens, and complement proteins (Table 1).

Effect of sea lamprey parasitism on the lake charr plasma proteome

Changes in protein abundances as a result of sea lamprey parasitism were considered for each wound Type from fish sampled immediately after parasitism, as well as the extent of recovery in fish sampled 7 months after receiving a Type A wound. Most changes occurred in fish with Type A wounds sampled immediately after parasitism, and all DA proteins could be functionally classified into immune response, transport, and blood coagulation. The recovery of DA proteins to non-parasitized control levels after 7 months was classified as either complete (ie. NPy = Ay and Ax $\neq$ Ay), partial (ie. NPy = Ay and Ax = Ay) or not recovered (NPy $\neq$ Ay and Ax $=$ Ay).

Table 1
Top 30 most abundant proteins in male siscowet lake charr (*Salvelinus namaycush*), ranked in decreasing order of abundance. Proteins identified as "uncharacterized" in the Uniprot *Salmonidae* database were matched to homologous proteins using Blastp, and high confidence matches (>80% identity) are provided in parentheses.

#	Accession	Protein Name
1	Q9PU67|Q9PU67_SALNM	Serotransferrin
2	A0A1S3N9Y0|A0A1S3N9Y0_SALSA	Fibrinogen alpha chain
3	A0A1S3LZJ4|A0A1S3LZJ4_SALSA	Myosin heavy chain, striated muscle-like isoform X1
4	Q70SU7|Q70SU7_SALAL	Salarin protein
5	S6A7U3|S6A7U3_SALLE	Phosvitin
6	P98093|CO3_ONCMY	Complement C3
7	A0A1S3R078|A0A1S3R078_SALSA	Alpha-2-HS-glycoprotein-like
8	A0A060YRH9|A0A060YRH9_ONCMY	Apolipoprotein H
9	A0A1S3KK24|A0A1S3KK24_SALSA	Histidine-rich glycoprotein-like
10	A0A4W5M443|A0A4W5M443_9TELE	Fibrinogen beta chain
11	A0A060ZVC3|A0A060ZVC3_ONCMY	Uncharacterized (Apolipoprotein A-II)
12	A0A1S3KKE9|A0A1S3KKE9_SALSA	Histidine-rich glycoprotein-like
13	A0A674EB86|A0A674EB86_SALTR	Uncharacterized (Hemopexin)
14	A0A1S3M7W8|A0A1S3M7W8_SALSA	Cerebellin-2-like
15	Q8JJ68|Q8JJ68_ONCMY	C-Type mannose-binding lectin
16	A0A1S3NAJ7|A0A1S3NAJ7_SALSA	Fibrinogen beta chain
17	A0A1S3MVQ2|A0A1S3MVQ2_SALSA	Hemopexin
18	O57523|APA11_ONCMY	Apolipoprotein A-I-1
19	A0A1S3SJI8|A0A1S3SJI8_SALSA	Plasma protease C1 inhibitor-like
20	P79825|P79825_ONCMY	Hemopexin
21	A0A4W5QLD0|A0A4W5QLD0_9TELE	Uncharacterized (no match)
22	A0A674B3H0|A0A674B3H0_SALTR	Uncharacterized (Complement C3)
23	A0A1S3PQV6|A0A1S3PQV6_SALSA	Hemopexin
24	A0A060YWV7|A0A060YWV7_ONCMY	Uncharacterized (Apolipoprotein A-II)
25	A0A673VKK2|A0A673VKK2_SALTR	Uncharacterized (Proteoglycan 4-like isoform X2)
26	A0A4W5NDM3|A0A4W5NDM3_9TELE	Hemopexin
27	A0A1S3SU51|A0A1S3SU51_SALSA	Saxitoxin and tetrodotoxin-binding protein 1-like
28	A0A060Y244|A0A060Y244_ONCMY	Uncharacterized (Hemopexin)
29	Q9PU68|Q9PU68_SALFO	Serotransferrin
30	A0A6F9CJX9|A0A6F9CJX9_9TELE	Uncharacterized (Apolipoprotein A-I)

Immune and transport proteins. Lake charr plasma had altered abundances of six proteins involved in immune and/or lipid transport functions as a result of sea lamprey parasitism. The largest observed change was an increase in complement factor B-like (A0A6F8ZXY2) immediately after parasitism in fish with Type A wounds relative to non-parasitized controls (log2FC = 0.857; P = 0.048), but its abundance in fish with Type B wounds was not significantly different from either non-parasitized controls or fish with Type A wounds. A partial recovery occurred after 7 months (ie. NPy = Ay, Ax = Ay), however considerable inter-individual variability in non-parasitized fish was evident (Fig. 2A). The abundance of a second immune protein, complement factor H-related protein 5-like isoform X2 (A0A674CVR4), was lower immediately after parasitism in fish with Type A wounds compared to non-parasitized control fish (log2FC = −0.287; P = 0.030) and fish with Type B wounds (log2FC = −0.332; P = 0.010). After 7 months, this protein was not different from non-parasitized control fish (NPy vs Ay: P = 0.200; Ax vs Ay: P = 0.060; Fig. 2B).

Two vitellogenin domain-containing proteins were decreased following sea lamprey parasitism. In fish with Type B wounds, the abundances of both proteins were significantly lower relative to non-parasitized control fish (A0A674EHA9: log2FC = −0.456, P = 0.050, Fig. 2C; A0A060W754: log2FC = −0.387, P = 0.048,

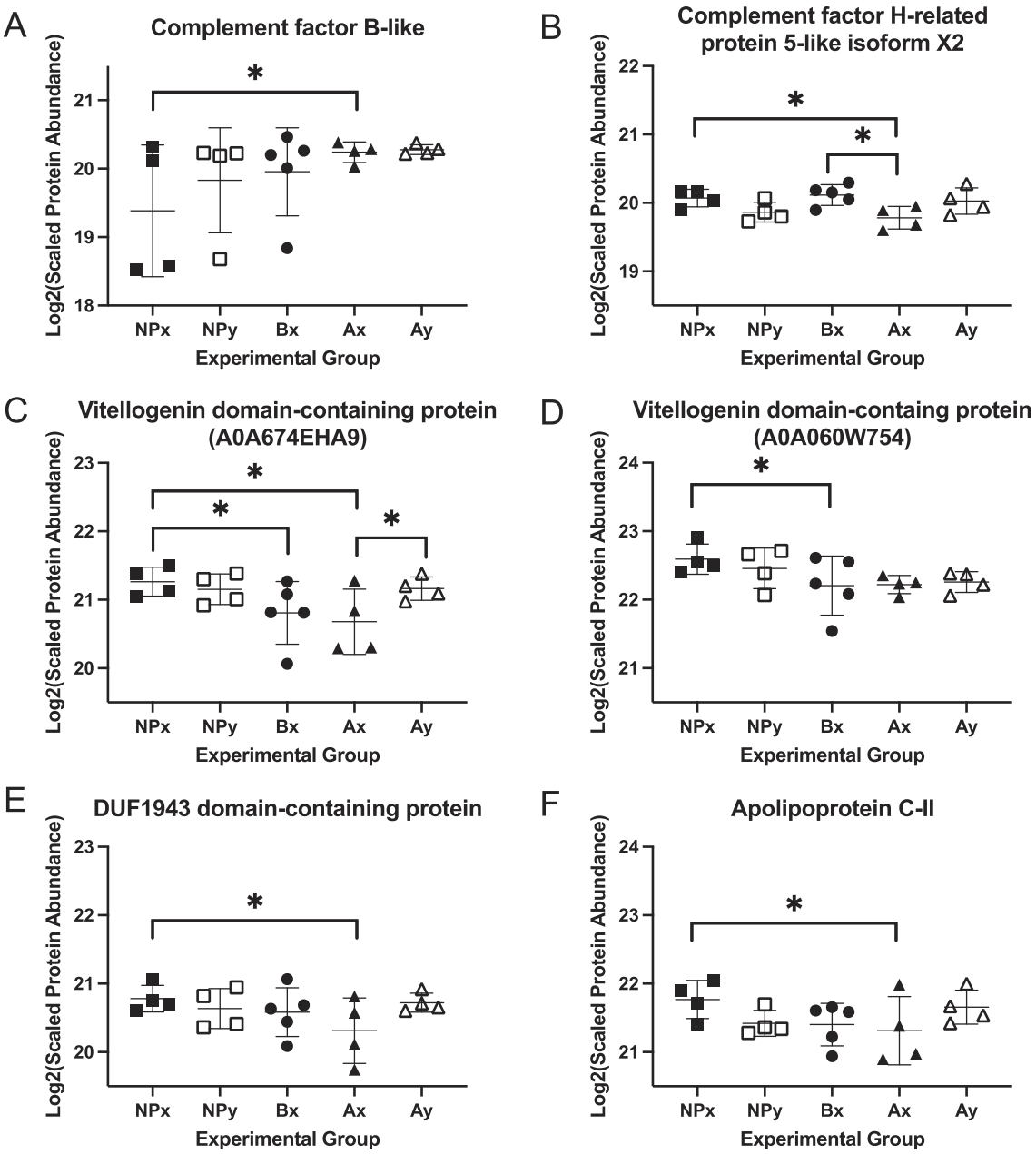

Fig. 2. Differentially abundant proteins that function in immune responses or transport, including two complement factors (A,B), two vitellogenin domain-containing proteins (C,D), the domain of unknown function (DUF) 1943 (a vitellogenin component protein; (E), and apolipoprotein C-II (F). Protein abundances were quantified in lake charr plasma collected from non-parasitized fish (NP, squares) and parasitized fish with Type B (B, circles) or Type A wounds (A, triangles). Blood collection occurred immediately after the parasitism event (x, closed symbols) and at 7-months post-parasitism (y, open symbols) on the same individuals. Protein abundances were compared across experimental groups using the DEP package in R, and an asterisk indicates a significant pairwise comparison (absolute log2FC > 0.263 and p < 0.05; n = 4–5). Individual data points are shown with line and whiskers to indicate mean ± SEM.

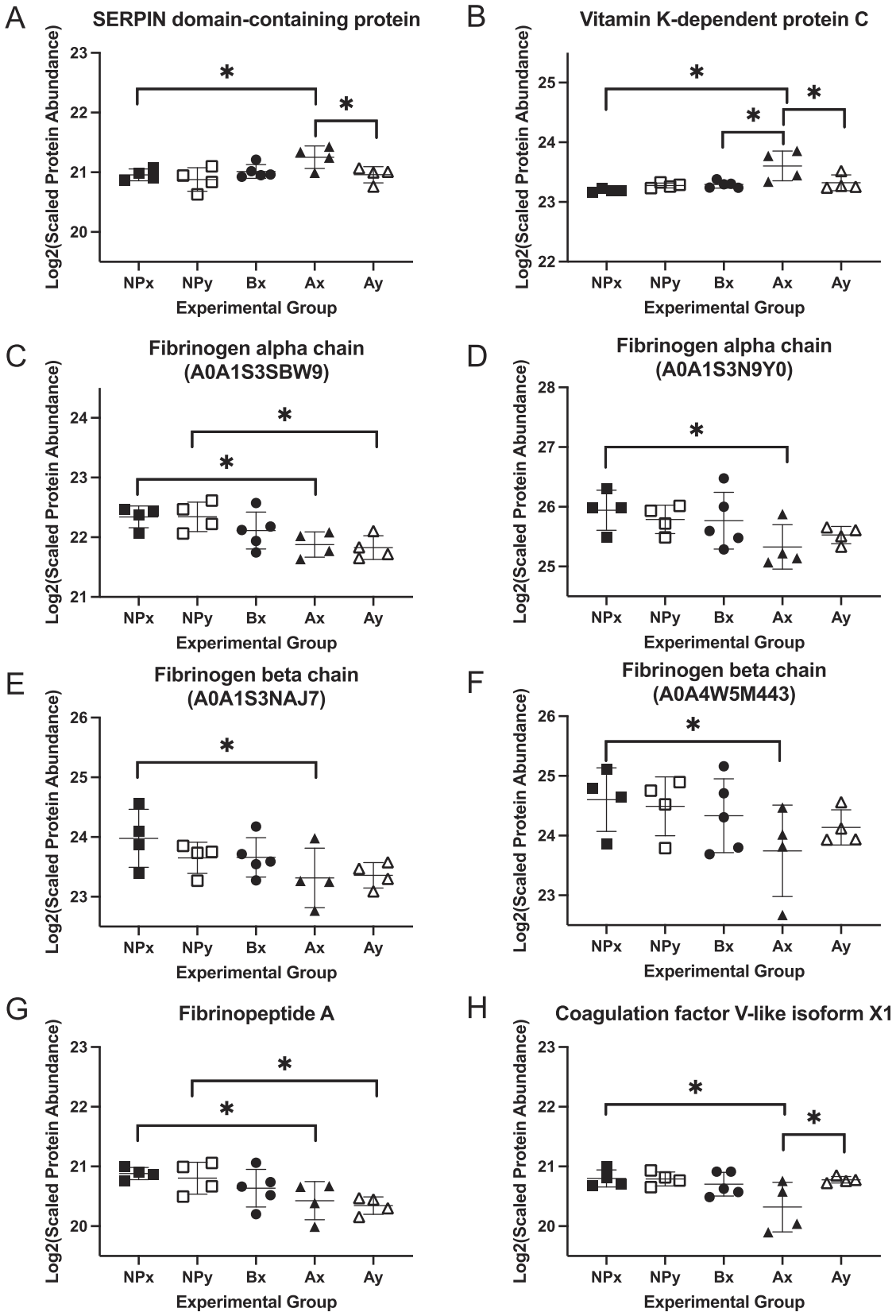

Fig. 3. Differentially abundant proteins that function in blood coagulation, including the anticoagulants (A) serine protease inhibitor (SERPIN) domain-containing protein and (B) vitamin K-dependent protein C, the fibrin clot component proteins fibrinogen alpha (C,D) and beta (E,F) chains as well as fibrinopeptide A (G), and a cofactor for thrombin activation coagulation factor V-like isoform X-1 (H). Protein abundances were quantified in lake charr plasma collected from non-parasitized fish (NP, squares) and parasitized fish with Type B (B, circles) or Type A wounds (A, triangles). Blood collection occurred immediately after the parasitism event (x, closed symbols) and at 7-months post-parasitism (y, open symbols) on the same individuals. Protein abundances were compared across experimental groups using the DEP package in R, and an asterisk indicates a significant pairwise comparison (absolute log2FC > 0.263 and $p < 0.05$; n = 4–5). Individual data points are shown with line and whiskers to indicate mean ± SEM.

Fig. 2D). In fish with Type A wounds, one of the vitellogenin proteins was significantly reduced immediately after parasitism (A0A674EHA9: log2FC = −0.585, P = 0.020) but recovered after 7 months (NPy vs Ay: P = 0.967; Ax vs Ay: P = 0.049; Fig. 2C). The other vitellogenin protein (A0A060W754) was reduced in fish with Type A wounds immediately after parasitism relative to non-parasitized fish, but this difference did not reach statistical significance (log2FC = −0.371; P = 0.070; Fig. 2D). The domain of unknown function (DUF) 1943 domain-containing protein (A0A060WCH3) was lower in fish with Type A wounds relative to non-parasitized controls (log2FC = −0.471; P = 0.041) but unaltered in fish with Type B wounds, and partially recovered after 7 months (NPy vs Ay: P = 0.691; Ax vs Ay: P = 0.071; Fig. 2E). Finally, the abundance of apolipoprotein C-II (A0A1S3RXS9) decreased in fish with Type A wounds relative to non-parasitized controls (log2FC = −0.456; P = 0.048), with intermediate levels in fish with Type B wounds (NPx vs Bx: P = 0.091; Ax vs Bx: P = 0.665). After 7 months, apolipoprotein C-II was partially recovered in fish that received Type A wounds relative to non-parasitized controls (NPy vs Ay: P = 0.286; Ax vs Ay: P = 0.127; Fig. 2F).

A seventh DA protein was elevated in fish with Type A wounds relative to non-parasitized controls (log2FC = 0.702; P = 0.030) and fish with Type B wounds (log2FC = 0.792; P = 0.012). This protein could not be confidently identified but may have immune function as it shared low homology with an Ig-like protein (uncharacterized protein; ESM Table S1).

Blood coagulation. Lake charr blood collected after sea lamprey parasitism had increased abundances of two anticoagulant proteins relative to non-parasitized control fish, and this response was more pronounced in fish with Type A wounds than Type B wounds. The first protein was a serine proteinase inhibitor (SERPIN) domain-containing protein (Fig. 3A), which was higher in fish with Type A wounds immediately after the parasite was removed relative to non-parasitized control fish (log2FC = 0.295, P = 0.001). This change was fully recovered 7 months later (NPy vs Ay: P = 0.506; Ax vs Ay: P = 0.023). The relative abundance of SERPIN domain-containing protein in fish with Type B wounds was intermediate, as it was not significantly different from non-parasitized controls but also did not meet the fold-change cut-off to be considered DA relative to fish with Type A wounds, despite a significant p-value (Bx vs Ax: log2FC = 0.239, P = 0.046). The second anticoagulant protein was vitamin K dependent protein C, which was found at higher levels in blood from fish with Type A wounds relative to both non-parasitized controls and fish with Type B wounds sampled immediately after parasitism (NPx vs Ax: log2FC = 0.411, P = 0.001; Bx vs Ax: log2FC = 0.311, P = 0.06), but fully recovered by 7 months (NPy vs Ay: P = 0.680; Ax vs Ay: P = 0.016; Fig. 3B).

Concomitant with changes in anticoagulant proteins, six proteins directly involved in the blood clotting cascade were decreased in fish with Type A wounds relative to non-parasitized control fish immediately after the parasitism event. These included two fibrinogen alpha chain proteins (A0A1S3SBW9: log2FC = −0.463, P = 0.012, Fig. 3C; A0A1S3N9Y0: log2FC = −0.614, P = 0.015, Fig. 3D), two fibrinogen beta chain proteins (A0A1S3NAJ7: log2FC = −0.663, P = 0.015, Fig. 3E; A0A4W5M443: log2FC = −0.858, P = 0.030, Fig. 3F), fibrinopeptide A (log2FC = −0.454, P = 0.018, Fig. 3G), and coagulation factor V-like isoform X1 (log2FC = −0.480, P = 0.006, Fig. 3H). These same six proteins were also lower in fish with Type B wounds relative to non-parasitized controls (max log2FC = −0.318), resulting in abundance levels that were not significantly different from either control fish or fish with Type A wounds. Following the 7-month recovery period, only coagulation factor V fully recovered to a level similar to non-parasitized control fish (NPy vs Ay: P = 0.925; Ax vs Ay: P = 0.009). Three clotting proteins partially recovered, such that circulating levels were not different from either non-parasitized controls or fish with Type A wounds immediately after parasitism (fibrinogen alpha chain A0A1S3N9Y0: NPy vs Ay: P = 0.269, Ax vs Ay: P = 0.396, Fig. 3D; fibrinogen beta chain A0A1S3NAJ7: NPy vs Ay: P = 0.252, Ax vs Ay: P = 0.862, Fig. 3E; fibrinogen beta chain A0A4W5M443: NPy vs Ay: P = 0.350, Ax vs Ay: P = 0.297, Fig. 3F). The remaining two proteins remained depressed after 7 months recovery relative to non-parasitized fish (fibrinogen alpha chain A0A1S3SBW9: NPy vs Ay: P = 0.006, Ax vs Ay: P = 0.767, Fig. 3C; fibrinopeptide A: NPy vs Ay: P = 0.016, Ax vs Ay: P = 0.636, Fig. 3G).

A modified clottability assay was used to determine whether plasma collected from parasitized lake charr would form a smaller fibrin clot than plasma from non-parasitized fish, as predicted by the reduced abundances of several fibrinogen subunits. Following addition of an excess of purified thrombin, lake charr plasma produced fibrin clots ranging from 7 to 1568 mg dry weight. A significant treatment effect was observed (Kruskal-Wallis test, P = 0.049) and clot weight was noticeably reduced in plasma from lake charr

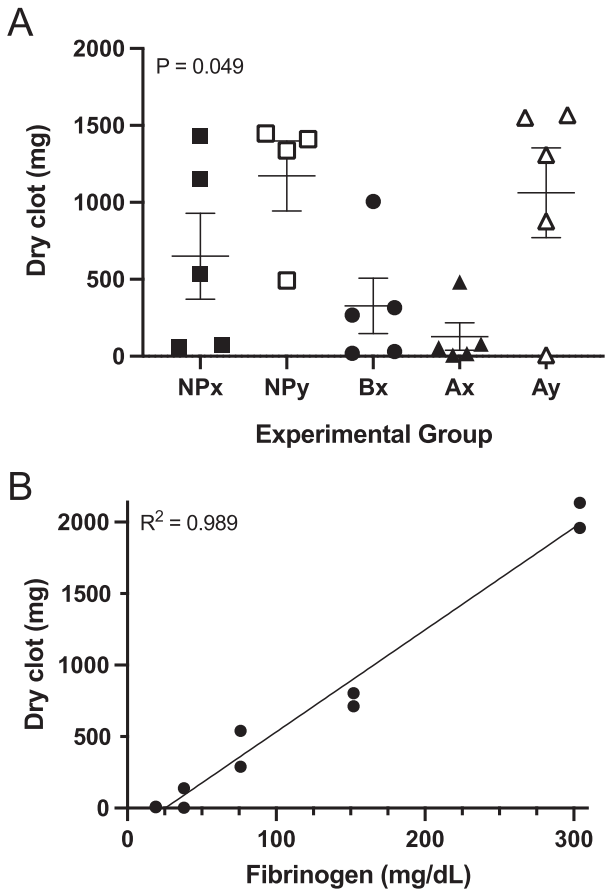

Fig. 4. Comparison of dry clot weights derived from a modified clottability assay. Fibrin clots were formed by incubating plasma with excess purified thrombin. (A) Dry clot weights of lake charr plasma collected from non-parasitized fish (NP, squares) and parasitized fish with Type B (B, circles) or Type A wounds (A, triangles). Blood collection occurred immediately after the parasitism event (x, closed symbols) and at 7-months post-parasitism (y, open symbols). Differences between experimental groups were determined using a Kruskal-Wallis test (P = 0.049, n = 5), but post-hoc testing did not reveal any pairwise differences. Individual data points are shown with line and whiskers to indicate mean ± SEM. (B) The clottability assay performed on serial dilutions of human reference plasma indicated a strong correlation between dry clot weight and fibrinogen concentration (mg/dL).

sampled immediately after parasitism (Ax, 128 ± 89 mg; Bx, 328 ± 180 mg) relative to other experimental groups (NPx, 650 ± 279 mg; NPy, 1172 ± 228 mg; Ay, 1062 ± 292 mg); but pairwise comparisons did not reveal specific differences between experimental groups (Fig. 4A). A strong correlation between starting plasma fibrinogen concentration and dry clot weight was observed when this assay was performed on human reference plasma (R^2 = 0.989; Fig. 4B).

Discussion

This paper presents novel data on protein-level changes in lake charr blood plasma after a lab-controlled sea lamprey predation event that resulted in either Type A or Type B wounds inflicted on host fish. Fish with Type A wounds were resampled 7-months later to assess recovery and latency of changes in the plasma proteome. While changes in a few proteins related to immune function and lipid transport were identified, the major finding of this study was that host fish with Type A wounds may experience a reduced capacity for blood clot formation, owing to a combination of changes in eight proteins involved in the clotting cascade (Fig. 5). In addition, the abundances of these same proteins in host fish with Type B wounds were intermediate between non-parasitized controls and fish with Type A wounds, and some changes persisted in the fish with Type A wounds for at least 7 months following the predation event. Therefore, this study offers proof of concept that protein biomarkers of sea lamprey parasitism are present in host blood and may help distinguish wound Type even months after the attack occurred. Moreover, decreased abundances of several fibrinogen subunits and a smaller thrombin-induced clot weight were observed in fish sampled immediately after the sea lamprey parasite was removed. This supports the hypothesis that plasma fibrinogen or blood clottability may prove useful in verifying mark classifications made in the field to improve the accuracy of marking data and, ultimately, enhancing the effectiveness of sea lamprey control efforts.

Effects of parasitism on immune and transport proteins

Ectoparasites have a unique relationship with their hosts in that they must feed while combatting the host's defenses and without compromising the immediate survival of the host. For example, an ectoparasite may actively suppress itching sensations and inflammatory responses of the host to ensure it remains attached and able to feed for an extended time; but a broadscale suppression of the immune system could leave the host susceptible to other pathogens which would not be of long-term benefit to the parasite (Wikel, 1999). In parasitic lampreys, bioactive components of lamphredin have been shown to suppress immune responses in the host, including neutrophil inhibition and T lymphocyte proliferation (Li et al., 2018; Sun et al., 2010; Xue et al., 2011). RNAseq analysis of liver samples collected from charr after about 4 d of sea lamprey parasitism did not show a transcriptomic response for genes commonly associated with pathogen exposure, but proinflammatory genes were altered (Goetz et al., 2016). In the present study, two complement proteins were oppositely affected by sea lamprey parasitism in fish with Type A wounds, but no other classic immune response proteins were identified as altered in parasitized fish. It is likely that the feeding time and extent of wounding are major factors in the dynamics and magnitude of the host response, and this may explain why stronger evidence of lamprey-induced changes in host immune proteins was not observed in the present study. At the same time, typical immune modulators that are expected of other pathogens (ex. bacteria, viruses) may not be recruited for sea lamprey parasites. For example, DUF1943 (Sun et al., 2013), vitellogenins (Garcia et al., 2010; Li et al., 2009, 2008; Liu et al., 2011, 2009; Zhang et al., 2011) and apolipoproteins (Dietrich et al., 2015; Tian et al., 2019) have all been described in immune-related functions in fish and were all

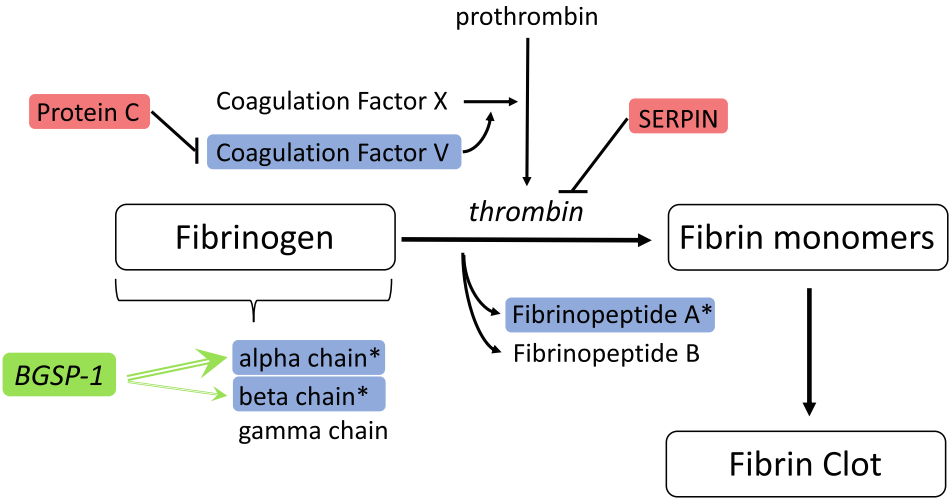

Fig. 5. Schematic depicting a simplified pathway of fibrin clot formation in vertebrates to demonstrate the impacts of sea lamprey parasitism on this process in lake charr plasma. Thrombin initiates clot formation by cleaving fibrinopeptides A and B from the alpha and beta chains of fibrinogen, respectively. The resulting free fibrin monomers can then polymerize into a fibrin clot. Thrombin is activated from prothrombin by coagulation factor X and its cofactor, coagulation factor V. Inhibition of blood clot formation is mediated in part by a serine protease inhibitor (SERPIN) that acts on thrombin, and protein C acting on coagulation factor V. Proteins highlighted in red were elevated in parasitized fish, while proteins highlighted in blue were lower in parasitized fish relative to non-parasitized controls. An asterisk indicates a protein that was not fully recovered to control levels 7 months after the parasitism event. Buccal gland secretory protein 1 (BGSP-1; green) is a fibrinogenolytic component of lamphredin secreted by parasitic lampreys during feeding. Green arrows show known specific targets of BGSP-1, with size indicating relative specificity (Xiao et al., 2007). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

impacted by sea lamprey parasitism. Furthermore, fish red blood cells are known to function as important immune cells in fish (Morera et al., 2011). and these were not included in the present analysis.

Sea lamprey parasitism carries an implicit energetic burden for the host while its blood is being consumed as well as during tissue rebuilding and healing once the parasite detaches. Not surprisingly, transcriptional alterations in several bioenergetic pathways result from sea lamprey parasitism in the lake charr liver (Goetz et al., 2016). Interestingly, ecomorph-specific responses were observed that correspond with the natural differences in energy storage between leans and siscowets (Goetz et al., 2014). More specifically, whereas leans are characterized by greater muscle glycogen stores and parasitism-induced changes in liver gene expression related to glycolysis and gluconeogenesis, siscowets tend to have relatively greater muscle lipid stores and more responsive transcriptional regulation of lipid metabolism in response to parasitism (Goetz et al., 2016, 2014, 2010). In line with this lipid-centric view of the siscowet ecomorph, the present study found reductions in four circulating proteins involved in lipid transport in fish with Type A wounds, including apolipoprotein C-II which could influence lipoprotein metabolism (Wolska et al., 2017), and three vitellogenin component proteins. One of the vitellogenin components was also significantly reduced in host fish with Type B wounds. Vitellogenins are large phospholipoglycoproteins that are comprised of three domains: the vitellogenin N domain, DUF1943 and the von Willebrand factor Type D domain (Carducci et al., 2019; Sun et al., 2013). Vitellogenins are principally known as egg-yolk precursors that circulate at high levels in female fish during ovarian maturation. The role of vitellogenins in oocyte maturation and their quantification in male fish as a biomarker of environmental estrogen exposure dominates the literature (Hara et al., 2016); however, the detection of vitellogenin domains in the plasma of male fish in the absence of any known estrogen stimulus (e.g. present study; Simmons and Sherry, 2015) encourages further investigation into its emerging non-reproductive roles (Carducci et al., 2019). The broader and long-term implications of the decreased abundances in these lipid transport proteins, including their direct influence on energy and metabolism, remain to be shown.

Anticoagulant effects of sea lamprey parasitism

Blood coagulation is a critical and conserved trait of the animal circulatory system that helps minimize blood loss and protect against infection after an injury (Doolittle and Surgenor, 1962; Herrick et al., 1999; Jagadeeswaran and Sheehan, 1999). Briefly, the common pathway for blood clot formation begins with the conversion of prothrombin to thrombin by the enzyme factor Xa and its cofactor, factor V. Activated thrombin cleaves fibrinopeptides A and B from the amino-terminals of the alpha and beta polypeptide chains of the circulating glycoprotein, fibrinogen. Once freed, the fibrin monomers immediately begin to polymerize resulting in the formation of a stable and insoluble fibrin clot. The fibrin clot helps to prevent blood loss and acts as an adhesive site that is crucial for platelet (ex. human) or thrombocyte (ex. fish) aggregation. Blood coagulation is tightly regulated by circulating anticoagulants, including members of the SERPIN family of enzyme inhibitors (Herrick et al., 1999; Huntington, 2011), as well as protein C which inactivates factor V to prevent thrombin activation and subsequent initiation of the clotting cascade (Griffin et al., 2007). In the present study, over half of the differentially abundant proteins found in fish with Type A wounds immediately after sea lamprey parasitism function in this clotting cascade (Fig. 5), including increases in a SERPIN domain-containing protein and vitamin K dependent protein C, as well as decreases in two fibrinogen alpha chains, two fibrinogen beta chains, fibrinopeptide A, and coagulation factor V-like (factor V). In host fish with Type B wounds, the abundances of all of these proteins were not different from either non-parasitized control fish or fish with Type A wounds, suggesting a graded response according to wound severity. Importantly, plasma from parasitized fish sampled immediately after the attack formed a smaller fibrin clot than plasma from non-parasitized fish. Given that fibrinogen is the precursor of a fibrin clot, and that initial fibrinogen concentration in human reference plasma is strongly correlated to dry clot weight, results from the clottability assay offer indirect evidence to cross-validate the reduction in fibrinogen measured using iTRAQ. Taken together, these results predict a decreased capacity for parasitized lake charr to stave blood flow from a wound, which would in turn support continued blood ingestion by an attached sea lamprey parasite.

The paired buccal glands of lamprey parasites produce and secrete a substance called lamphredin when feeding on host fish. Among its many properties, lamphredin is known as a potent anticoagulant with fibrinogenolytic activity that promotes continuous blood flow to the wound site (Baxter, 1956; Gage and Gage-Day, 1927; Lennon, 1954; Xiao et al., 2007). Indeed, anticoagulant secretions are common among ectoparasites like ticks and leeches (Basanova et al., 2002; Zavalova et al., 2002), as well as in striking predators like brown spiders (Zanetti et al., 2002) and venomous snakes (Daoud et al., 1986; Jagadeesha et al., 2002; Maruyama et al., 1992; Siigur and Siigur, 1991; Tseng et al., 1989). In the Arctic lamprey (*Lethenteron camtschaticum*; formerly the Japanese lamprey, *Lampetra japonica*), one of the most prevalent proteins in lamphredin is buccal gland secretion protein 1 (BGSP-1), which has potent fibrinogenolytic activity against the alpha chain of human fibrinogen, as well as significant but lower specificity for fibrinogen beta chain (Xiao et al., 2007). Although the specific composition of lamphredin from *P. marinus* is undescribed, the presence of a fibrinogenolytic protein like BGSP-1 would explain the observed decreases in fibrinogen polypeptides and fibrinopeptide A in lake charr with Type A wounds. Interestingly, none of the fibrinogen component peptides fully recovered after 7 months, and two showed no recovery (fibrinogen alpha chain A0A1S3SBW9, fibrinopeptide A). As lamphredin is unlikely to persist in the host circulation once the parasite has detached, results of this study suggest a downregulation of fibrinogen synthesis may be induced by the sea lamprey attack. Sea lamprey parasitism induces a sizeable transcriptional response in the liver of lake charr, but analysis of RNAseq data from parasitized lean and siscowet ecomorphs did not reveal changes in fibrinogen transcripts or other aspects of the clotting response (Goetz et al., 2016). Therefore, long-term reductions in circulating fibrinogen may be a result of post-transcriptional regulation in the liver of parasitized fish, but this remains to be determined. Irrespective of mechanism, it will be important to determine the latency of the anticoagulant response to sea lamprey parasitism as well as its physiological consequences.

In contrast to the fibrinogen component proteins, the abundances of SERPIN domain-containing protein, vitamin K dependent protein C, and coagulation factor V-like isoform X1 in lake charr with Type A wounds all fully recovered to levels similar to non-parasitized control fish after 7 months. This suggests a direct link between the physical presence of the sea lamprey parasite and the abundance of these proteins. Given the spectrum of bioactive compounds present in lamprey buccal gland secretions (Sun et al., 2010; Xiao et al., 2012, 2007), it is likely that some components of lamphredin are responsible for the broad disruption of clotting cascade proteins observed in this proteomics study.

Biomarker potential and management implications

Mark assessment on lake charr remains a critical component of sea lamprey monitoring in the Great Lakes and helps determine where to direct sea lamprey control efforts each year. The success of these programs is critical to ensuring that sea lamprey numbers remain low to minimize their impact on host fish populations. The main objective of this study was to determine whether protein biomarkers of sea lamprey parasitism can be detected in lake charr blood plasma, with the rationale that a robust and quantitative indicator of parasitism could augment sea lamprey mark assessment efforts. Specifically, the inclusion of an objective index of parasitism alongside the more subjective classification of wound Type and healing stage is expected to improve the accuracy of sea lamprey population and impact estimates, ultimately leading to more effective application of control measures in the Great Lakes. Accordingly, the best blood biomarkers should: (i) be specific to sea lamprey parasitism and largely independent of other environmental factors; (ii) be robust across sex, life stage, and/or ecomorph of the host; and (iii) vary in a predictable and quantitative manner with the dynamics of a sea lamprey attack and/or the prognosis of the host. While observed changes in vitellogenin domain proteins suggest a quantitative connection to sea lamprey parasitism, it is unlikely that such proteins would pass validation efforts owing to the strong likelihood that circulating vitellogenin levels are dependent on the sex and maturation of the host, as suggested by seasonal variation in gonadosomatic index (Goetz et al., 2011). However, observed changes in several proteins involved in fibrin clot formation support quantification of blood clotting capacity in host fish as a viable metric of sea lamprey parasitism. The concerted response of multiple effectors in the coagulation cascade for fish with Type A wounds, in conjunction with a partial or incomplete recovery in many of these proteins after 7 months, suggests that the clotting response would be a sensitive measure for confirming Type A wounds even after healing is initiated. Moreover, the response of these same proteins in host fish with Type B wounds was intermediate between non-parasitized controls and fish with Type A wounds, suggesting that the influence of sea lamprey parasitism on the clotting response may vary quantitatively with attack intensity. Importantly, it is unlikely that effects on blood coagulation arising from sea lamprey parasitism would vary between male and female lake charr, or among lake charr ecomorphs, and may even apply to other host species. Therefore, results of the present study encourage additional efforts to validate the quantification of blood coagulation as a bioindicator of sea lamprey parasitism.

Quantifying blood coagulation as a biomarker is not a new concept, and offers a simple, fast, and cost-effective means to assess an animal's capacity to form blood clots. For example, clotting assays are common in clinical settings for diagnosing a broad range of hematological disorders related to hyper- and hypo-coagulation (Duvoix et al., 2013; Falanga and Marchetti, 2018; Klim et al., 2018; Miesbach et al., 2010). In addition, clotting assays have been effective in detecting exposure to anticoagulant rodenticides in birds of prey (Hindmarch et al., 2019). Established methods for quantifying the coagulation cascade rely on the direct or indirect measurement of fibrin clot formation. For example, following addition of purified thrombin to a plasma sample, the Clauss assay measures time to clot formation while the prothrombin (PT) time assay measures a change in optical density as the fibrin clot forms. While both the Clauss and PT-time assays are widely used in clinical applications due to their sensitivity for detecting low fibrinogen concentrations, their reliance on a time variable may pose a challenge for samples collected with heparin (Ogawa et al., 2015) or when species mismatches in thrombin and fibrinogen exist (Doolittle et al., 1962). In contrast, clottability assays circumvent these issues by quantifying the resulting fibrin clot after a standardized amount of time, when clot weight or clot protein content is directly related to initial fibrinogen concentration since most of the protein in the resulting clot is fibrin (Mackie et al., 2003). Nevertheless, the principles of blood coagulation can be universally applied which will facilitate optimizing a sensitive clotting assay for fish blood. Important next steps are to validate blood clotting as an endpoint against a range of potentially confounding variables present in field-collected samples, including the duration of parasite feeding, relative biometrics of the parasite and host (ex. size, condition factor), and host biology (ex. age, sex, ecomorph). If variation in blood clotting can be used as a reliable bioindicator of sea lamprey parasitism in lake charr, this could offer a quick, cost-effective, and efficient tool for validating wound classifications, which are subjective and error-prone (Firkus et al., 2020).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

OMNB analyzed data, prepared figures, and wrote the first manuscript draft; TJF conducted the parasitism trials as part of a larger and separate study funded by the Great Lakes Fishery Commission Sea Lamprey Research Program, and provided editorial feedback; FWG raised the siscowet hosts and provided editorial feedback; CAM conceived the project concept, contributed to experimental design of the current study and original parasitism trials, and provided editorial feedback; SLA contributed to experimental design of the current study, performed the proteomics experiment, analyzed data, and provided editorial feedback. All authors approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jglr.2021.11.005.

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