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Smell of Infection: a novel, non-invasive method for detection of fish excretory-secretory proteins

Rebecca J. Pawluk[1*], Rebekah Stuart[2], Carlos Garcia de Leaniz[1], Joanne Cable[4], Russell M. Morphew[3], Peter M. Brophy[3], Sofia Consuegra[1]

[1]Swansea University, College of Science, Biosciences, Swansea, SA2 8PP, UK

[2]Wales Veterinary Science Centre, Buarth, Aberystwyth, Ceredigion, SY23 1ND, UK

[3]Aberystwyth University, IBERS, Penglais, Aberystwyth, Ceredigion, SY23 3FL, UK

[4]Cardiff University, School of Biosciences, Cardiff, CF10 3AX, UK

*Correspondence to rebeccajane93@hotmail.co.uk

Abstract

Chemical signals are produced by aquatic organisms following predatory attacks or perturbations such as parasitic infection. Ectoparasites feeding on fish hosts are likely to cause release of similar alarm cues into the environment due to the stress, wounding and immune response stimulated upon infection. Alarm cues are often released in the form of proteins, anti-microbial peptides and immunoglobulins that provide important insights into bodily function and infection status. Here we outline a non-invasive method to identify potential chemical cues associated with infection in fish by extracting, purifying and characterizing proteins from water samples from cultured fish. Gel free proteomic methods were deemed the most suitable for protein detection in saline water samples. It was confirmed that teleost proteins can be characterised from water and that variation in protein profiles could be detected between infected and uninfected individuals and fish and parasite only water samples. Our novel assay provides a non-invasive method for assessing the health condition of both wild and farmed aquatic organisms. Similar to environmental DNA monitoring methods, these proteomic techniques could provide an important tool in applied ecology and aquatic biology.

KEYWORDS: Alarm cues, gel free MS, *Kryptolebias marmoratus*, odour, parasitic infection

1. Introduction

Chemical cues released into the environment following natural perturbations, predator attacks or social threats allow animals to assess potential dangers and may lead to increased survival chances (1). In the aquatic environment, cues released in the form of soluble pheromones or as chemicals following physical trauma to the epidermis are described as alarm cues (2). These cues can signal the presence of danger, including the scent of predatory species in the environment (disturbance cues) (3, 4) or the odour of parasitized conspecifics (5). Additionally, innate immune responses activated upon pathogen infection can cause variation in body odour; this has been documented in humans for example, whereby endotoxin-exposed individuals smelt significantly more unpleasant than their control counterparts indicating a social cue of sickness (6). Changes in body odour have also been seen in infected mice, where females prefer the urinary odours of uninfected male individuals (7). Yet, studies that describe infection odours in aquatic environments are scarce (8, 9), despite the importance of chemical communication, particularly in potentially turbid conditions (10).

Chemical cues also allow individuals to recognise neighbours, assess social hierarchies, and determine mating ability by genotype matching, which may translate into attraction or conspecific avoidance (11, 12). Odour based mate choices have been linked to the Major Histocompatibility Complex (MHC) and its associated peptides (13) and allow individuals to discriminate between MHC genotypes on the basis of variation in immune-related peptides released into the water (13). This enables mate choice to maximise the immuno-competence of offspring (14), and MHC-related peptides are likely used for relatedness discrimination in several species e.g. *Salvelinus alpinus* (15) and *Gasterosteus aculeatus* (13). Analyses of social communication in mice has also provided evidence that a variety of polymorphic proteins in urine (major urinary proteins) provide a unique identity to the owner and are used as scent signals (16).

Many teleost fish can produce and perceive water-borne cues released by stressed conspecifics, including free cortisol (17) and alarm cues released by specialized skin cells when individuals are injured (18). Zebrafish (*Danio rerio*), for example, are able to detect cues in the water from physically stressed individuals and avoid them (1) and Atlantic salmon can detect when a predator has consumed conspecifics (19). Similarly, Nile tilapia (*Oreochromis niloticus*) display anti-predatory behaviours (including freezing and dorsal fin erection) in response to water conditioned with conspecific skin extract that mimics a predatory attack (20). Alarm cues also arise from parasitic infections, particularly ectoparasites that cause trauma to the epidermis initiating immune responses in a similar way to predator attacks (21, 22). Argulid parasites attach to host fish by large maxillary suckers and feed on host blood and skin (23), this type of parasite feeding causes skin wounding, ulcers and increases the susceptibility of the host to secondary infections (24). Immune responses in fish skin, largely identified in the mucosal layer include several components that reduce pathogen and allergen entry (25). Such components consist of antimicrobial peptides and enzymes (such as lysozymes, phosphatases and proteases) for enhanced protection and wound healing (26-28), lectins and proteins that enhance pathogen expulsion and immune activation (25, 29, 30) and immunoglobulin antibodies for protection against surface infections (31). It is on this basis that the current study aimed to investigate excretory-secretory proteins released by hosts and parasites upon infection.

Gel based and gel free proteomic techniques are often used to detect proteins from body tissues of an organism (32, 33) and are also useful for studying the excretory-secretory proteins produced by endo-parasitic helminths (34). For example, *Fasciola hepatica*, an economically important helminth of livestock is well studied, partly as it can easily be cultured in media, providing a clearer understanding of host-parasite communication and host manipulation (35-37). Fewer studies use *in vitro* culturing techniques to obtain proteins from ectoparasitic

organisms; culturing of such parasites commonly involves rearing host teleost species under laboratory conditions followed by subsequent infection of these hosts via anaesthesia (46). Despite difficulties associated with culturing these parasites (38), such systems provide a unique chance to collect and analyse protein data from host-parasite interactions. Furthermore, there is a distinct lack of knowledge surrounding environmental proteins from fresh and marine water bodies and the potential use of proteins as a non-invasive detection method for infection. Research into meta-proteomics of water samples to date has focused on methods for marine sediments (e.g. 39). Yet, these methods have never been applied to teleost fish, particularly in brackish water samples or in relation to infection or parasite presence, which can be critical the early detection of infection in aquatic species.

Kryptolebias marmoratus (the mangrove killifish) is a small, naturally inbred, cyprinid fish species that is easily maintained under laboratory conditions. Isogeneity in this species via constant selfing makes it a useful model organism for the study of physiological plasticity and response to environmental stressors (40, 41). *Argulus foliaceus*, is a generalist fish parasite found throughout Europe that is known to cause epizootics and problems in both brackish and freshwater fish farms (42-45). Here we assessed the use of a novel non-invasive proteomic approach to identify fish infection by comparing proteins released into water by infected and uninfected *K. marmoratus*. Additionally, we used this study system to develop a proteomic method of potential application to assess health status and stress response in fish populations

2. Experimental Procedures

2.1 *Water sample collection, preparation and visualisation*

The study species, *K. marmoratus*, originated in Belize, from two self-fertilising lines (DAN and R), that have undergone >30 generations of selfing (46). Eighty size- matched *K. marmoratus* (12 to 14 months old) reared individually from hatching were selected from the

two lines (40 DAN and 40 R) and kept in individual aquaria (12 L x 8 W x 8.5 H cm) containing 750 ml of brackish water (15 ppt salinity, constituted from dechlorinated water and marine filtered water) under controlled conditions (12L:12D photoperiod, 24 ± 1^0C).

Twenty fish from each line were individually infected with one adult individual of the ectoparasitic crustacean *Argulus foliaceus*. The culture of *A. foliaceus* originated from carp (*Cyprinus carpio*) caught in a still water fishery in North Lincolnshire, July 2014, and thereafter was maintained on *Gasterosteus aculeatus* (three-spined sticklebacks) at Cardiff University as detailed in Stewart *et al*. (2017) (47). The other twenty fish from each line were kept as control individuals, as described in Pawluk (48). Two water samples for proteomic analyses (50 ml each) were collected from each aquarium (80 aquaria total) prior to filtration (Minisart 25 mm Pore size 0.2 µm filter) and storage at -80°C, the second sample acting as a spare. Twenty water samples (50 ml each) were also taken from containers of the same size containing a single *A. foliaceus* as a control for parasite proteins released into the water. Amicon filter units were used to reduce water samples from 36 ml (3 subsamples of 12ml) to approximately 2 ml (10KDa cut off, 5000 x g for 10 minutes, with two filter washes); subsequent quantification (Bradford, 49) using Sigma Bradford Reagent (according to the manufacturer's instructions) and a Cary 50 Bio UV-visible spectrophotometer at 595 nm was completed. All samples were precipitated using the addition of 4 volumes of ice cold acetone followed by 1-hour incubation at -20°C. Proteins were then pelleted by centrifugation at 4^0C and 21000 x g for 15 mins. Pellets were then dried for 30 mins before being resuspended in 30 µl of Buffer Z (8 M Urea, 2% w/v CHAPS, 33 mM DTT, 0.5% ampholytes pH range 3-10). Subsequent 1D SDS-page was performed using polyacrylamide gels (12.5%); they were then fixed overnight (in 10% ethanol, 40% acetic acid) and silver stained (50). The molecular weight of protein bands identified on 1D gels were calculated using a standard curve of Log(Mw) versus the mobility shift (*Rf*).

2.3 *Trypsin digestion and gel free mass spectrophotometry*

Fifteen water samples were prepared using Amicon filtration, as described previously, prior to trypsin digest and mass spectrometry analysis (3 individual fish replicates per group: DAN infected, DAN control, R infected, R control and *Argulus* only). Sample aliquots of 100 µl each were added to 6 M urea, in 100 mM tris buffer, and then reduced using 5 µl reducing agent containing DTT and Tris stock (200 mM DTT, 100 mM Tris) for 1 hour. Subsequently, 20 µl alkylating agent (200 mM iodoacetamide and 100 mM Tris) was added to each sample and vortexed before 1 hour incubation at room temperature. A further 20 µl of reducing agent was added per sample and incubated at room temperature for 1 hour. Sample urea concentration was reduced (to approx. 0.6 M) by diluting each sample with 75 µl of water before mixing. Trypsin digestion began following the addition of trypsin solution to a final concentration of 50 ng/µl, which was subsequently mixed and then incubated overnight at 37°C. The reaction was stopped by adjusting the pH to >6 by adding concentrated acetic acid. All 15 samples were reduced to 100 µl aliquots using a speed vacuum prior to analysis liquid Chromatography tandem mass spectrometry using the Agilent 6550 iFunnel Q-TOF mass spectrometer with Dual AJS ESI source coupled to a 1200 series HPLC-Chip system (Agilent, Cheshire, UK).

The HPLC-Chip/Q-TOF system was equipped with a capillary loading pump (1200 series, Agilent Technologies) and a nano pump (1200 series, Agilent Technologies). Sample injection was conducted with a micro auto sampler (1100 series, Agilent Technologies), where 1 µl of sample in 0.1% formic acid was loaded on to the enrichment column at a flow of 2.5 µL/min followed by separation at a flow of 300 nL/min. A Polaris Chip was used (G4240-62030, Agilent Technologies), comprising a C18 enrichment/trap column (360 nl) and a C18 separation column (150 mm x 75 Âµm), where ions were generated at a capillary voltage of 1950 V. The solvent system was: solvent A (ultra-pure water with 0.1% formic acid), and solvent B (90% acetonitrile with 0.1% formic acid). The liquid chromatography was performed

with a piece-linear gradient using 3-8% of solvent B over 0.1 minutes, 8-35% solvent B over 14.9 minutes, 35-90% solvent B over 5 minutes and hold at 90% solvent B for 2 minutes. Tandem mass spectrometry was performed in AutoMS2 mode in the 300-1700 Da range, at a rate of 5 spectra per second, performing MS2 on the 5 most intense ions in the precursor scan. Masses were excluded for 0.1 min after MS/MS was performed. Reference mass locking was used for internal calibration using the mass of 391.2843 Da. Peak lists were generated with Mass Hunter Qualitative Analysis software (V B.06, Agilent Technologies) and exported as Mascot Generic Files.

2.4 *Data submission and assessment based on MASCOT scoring*

Mass spectral data from all 15 samples were submitted for database searching using the MASCOT program (Matrix Science Ltd., version 2.1). Search parameters allowed a maximum of one missed cleavage. Additionally, a fixed modification was set for cysteine at 161 Da and variable modifications tested for matches 0, 1, 2, or 3 oxidised methionine residues. Lastly, a set peptide tolerance of 1.2 Da and MS/MS tolerance of 0.6 Da were stipulated. Spectra were searched against the *K. marmoratus* transcriptome (48) as well as a recent version of the NCBI non-redundant protein database sequences. Both analyses resulted in a total of 30 protein lists that were subsequently sorted into treatment groups; each sample from each group was filtered for protein number above the MASCOT significance threshold at $p = 0.05$ (Transcriptome data ≥ 50, NCBI data ≥ 59), protein identified in all three replicates and proteins identified in two of three individuals for each treatment (Table S2 & S3). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (51) partner repository with the dataset identifier PXD010987.

2.5 *GO annotation, pathway analysis and statistical testing*

All proteins for each sample and treatment were considered to compare data searches and to complete a functional analysis (GO annotation, level GO ALL) of the results using the Database for Annotation, Visualization and Integrated Discovery, version 6.8 (DAVID) (52). Pathway analysis was completed using REACTOME pathways (53) for the three biogicial replicates of each group. Lastly, protein release for treatment groups was compared statistically using t-tests. MASCOT scores for samples from the two respective groups (Infected and control) were compared for specific proteins (OSER1, KRT94). Additionally the number of proteins related to specific functional groups (identified from DAVID) were compared for methylation and immune functions. All figures produced for data visualisation were created using R version 3.4.0 (54).

3. Results

3.1 *Protein visualisation and functional analyses*

Results from quantification and one dimensional SDS-page indicated the presence of proteins at approximately 61.6, 47.9, 42.3 and 24.8 kDa (Figure 1).

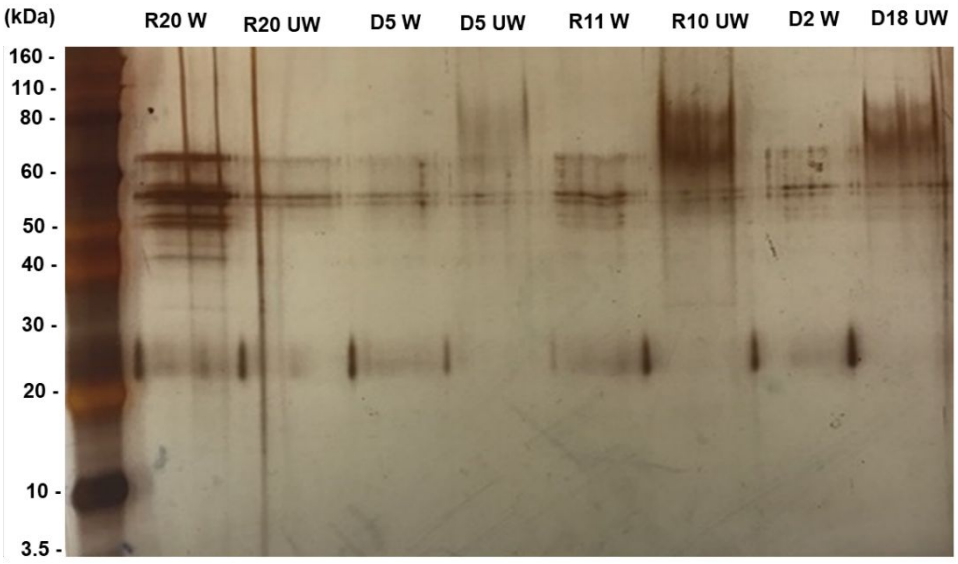

Figure 1. SDS-PAGE gel of the expression of proteins concentrated using Amicon filtration steps, from two strains (D: DAN and R: R) for unwashed (UW) and washed (W) filters.

As 1D gel electrophoresis successfully indicated purified proteins a gel free approach was taken to identify all proteins within these samples. Soluble proteins from water samples were identified using both the *K. marmoratus* transcriptome (48) (MASCOT score cut of ≥50) and from a search of the NCBI database (MASCOT score cut of >59). The total number of proteins identified for the *K. marmoratus* transcriptome and NCBI database ranged from 28 to 53 per sample. The percentage of identified proteins over the MASCOT cut off score for each data set ranged from 2.9 – 16.3% from transcriptome sourced data and 7.7-20.9% from NCBI sourced data (Table S1). Trypsin (used for digestion) and keratin, mostly deemed to be from human origin and thus contaminants were highly abundant (had a MASCOT score on or above the cut off) and overlapped between search methods (Transcriptome and NCBI) (Table S2 and S3).

Gene ontology analysis indicated that functional proteins involved with immune response and development (Figure S2 and S3) were identified in both data sets however on inspection of these proteins there was little overlap between search methods. Pathway analysis (Table 1, Table S5 and Figure S3) was completed for a comparison of enriched protein groups for all 5 groups (DAN control and infected, R control and infected, and *Argulus* only water). For all fish water samples (DAN and R, infected and control) proteins were mainly involved in metabolism, in three of these groups these proteins had the highest number of hits. Proportionally fewer proteins were reported in *Argulus* only samples when compared with the others and proteins from this group primarily had roles in transcription and homeostasis. Proteins with roles in cytokine signalling were shown to be in infected fish water samples but not in DAN.

Table 1. The top five entities identified from the REACTOME pathway analysis of 5 groups of water samples (from DAN infected, DAN control, R infected and R control *Kryptolebias marmoratus* as well as *Argulus* only samples).

Group	Pathway name	Entities found	Entities Total	Entities pvalue	Entities FDR	Reactions found
Dan infected	Metabolism	10	2,116	0.84	8.35E-01	34
	Immune System	6	2,226	1.00	9.95E-01	17
	Adaptive Immune System	3	944	0.93	9.28E-01	6
	Generic Transcription Pathway	3	1,152	0.97	9.73E-01	7
	RNA Polymerase II Transcription	3	1,274	0.99	9.86E-01	18
Dan control	Metabolism	6	2,116	1.00	9.97E-01	9
	Disease	4	1,148	0.95	9.52E-01	25
	Innate Immune System	4	1,180	0.96	9.59E-01	12
	Post-translational protein modification	3	1,415	1.00	9.97E-01	21
	Signaling by Receptor Tyrosine Kinases	2	471	0.82	8.22E-01	2
R infected	Metabolism	8	2,116	0.99	9.92E-01	23
	Immune System	8	2,226	1.00	9.96E-01	17
	Metabolism of proteins	6	2,111	1.00	9.99E-01	11
	Signal Transduction	6	2,738	1.00	1.00E+00	8
	Innate Immune System	5	1,180	0.94	9.41E-01	8
R control	Signal Transduction	10	2,738	0.98	9.84E-01	40
	Metabolism	8	2,116	0.96	9.62E-01	24
	Metabolism of proteins	5	2,111	1.00	9.98E-01	4
	Immune System	4	2,226	1.00	1.00E+00	15
	GPCR downstream signalling	3	1,146	0.98	9.77E-01	4
Argulus	Signal Transduction	7	2,738	1.00	9.99E-01	27
	Metabolism of proteins	6	2,111	0.99	9.91E-01	31
	Metabolism	5	2,116	1.00	9.97E-01	7
	Immune System	5	2,226	1.00	9.98E-01	21
	Gene expression (Transcription)	3	1,416	0.99	9.92E-01	5

3.2 *Protein identification and comparisons*

Analysis of proteins identified using the killifish transcriptome identified a similar number of proteins released (MASCOT ≥50) by all experimental groups (Table S1). Actin family beta (for cell motility, structure and integrity) and fish keratin (from host tissue) proteins were expressed in all treatment groups. OSER1, a protein with roles in oxidative stress response, was abundant in all fish water samples, but not in *Argulus* only samples. Despite this, no significant variations in MASCOT scores between control and infected water samples were identified for OSER1 ($t_{6.96}$=1.521, P=0.172). In addition to proteins detected from the killifish transcriptome, results were searched against the NCBI database to identify proteins from fish and parasite origins. Of the forty-seven proteins with a MASCOT score on or above the 59 cut off, 49% were deemed as human contamination (Table S3, in red) but 13% of proteins included claw keratin and proteins associated with teleost fish (polyserase-2-like proteins and Chymotrypsin-like protease proteins).

Variations between infected and uninfected fish samples were identified for both transcriptome and NCBI data sets. Proteins identified from the transcriptome indicated that Keratin 94 (of fish origin), associated with host tissues, was only identified in infected fish water samples. However, these samples did not differ significantly when MASCOT scores from each group were compared ($t_{6.9}$=0.88, P=0.396). Zinc finger proteins (a class of protein involved with nucleic acid binding and protein dimerization) were highly abundant in DAN infected and *Argulus* only samples.

Gene ontology analysis of proteins from the transcriptome was conducted and compared between treatments (Figure 2). This analysis indicated that proteins involved in pigmentation (TSPAN36 and SLC45A2) and binding were only detected among infected fish and no protein group was identified solely in the control groups. Previous work has demonstrated links

between infection and pigmentation (55, 56), therefore, proteins related to colour in *K. marmoratus* are likely promising indicators of immune response. All groups contained proteins related to metabolism, proteolysis and circulation. Finally, proteins for sound perception, cilium movement and mucus production (Figure 2A) were only identified in water from fish origin. Statistical analysis of gene ontology data from NCBI indicated that a significantly greater abundance of proteins for methylation were identified in infected fish water samples when compared with controls (t_5 = 2.397, P=0.042) as can be seen in figure 2. Conversely despite variation indicated in figure 2, the number of proteins with roles in immune response did not differ significantly between groups (t_5 =-0.682, P=0.522). For control groups, functional proteins identified were involved in apoptosis and autophagy. Lastly protein families observed in all groups included extracellular proteins, as well as proteins involved in the immune response, and in carbohydrate binding (for all see Figure 2A).

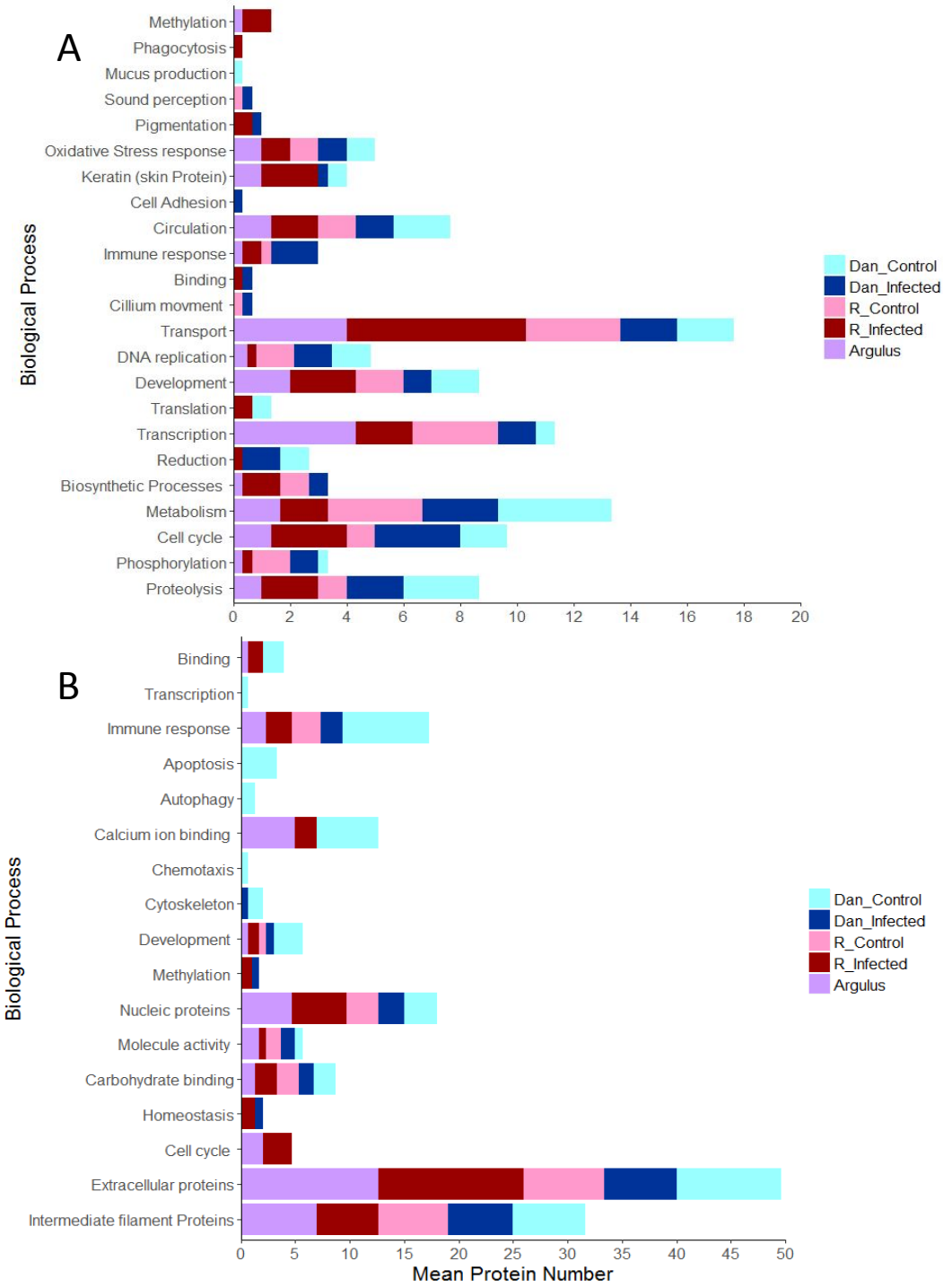

Figure 2. Go annotation of all proteins identified from infected (with *Argulus foliaceus*), uninfected and parasite only water samples for two lines (DAN and R) of *Kryptolebias marmoratus* transcriptome (A) and NCBI data (B).

4. Discussion

Current, non-lethal fish health monitoring involves collection and subsequent analysis of the external surface mucus of teleost species (57, 58). Fish skin mucosa acts as an important barrier against pathogens (59, 60) and within this barrier proteins may be secreted, synthesized and released (in the form of dead cells) leading to the production of identifiable immune related molecules (61). Release of molecules into the surrounding aquatic medium is thought to act as chemical stimulus that enable teleost fish to assess the health, genetic background and identity of conspecifics (11, 15). The methods used in the current study indicate that these proteins can be extracted, purified and characterized from teleost water samples and have the potential to inform infection status non-invasively, without the need for invasive sampling. In the current study, initial gel based methods demonstrated a number of observable protein bands that may be indicative of heat stress and heat shock proteins (62, 63), binding proteins (64) and keratins (65). Furthermore, gel-free methods following trypsin digestion of samples greatly increased the number of proteins identified and confirmed the presence of fish keratins, binding proteins and immune related molecules.

Skin extracts are thought to be present in the mucosa following skin rupture, leading to the release of alarm cues, identifiable by conspecifics (59, 66). Fish keratin proteins (and many actin proteins below the MASCOT cut-off) were identified in samples from infected fish only; these proteins are likely to originate from the skin surface (due to the structural function of keratin) in the form of ruptured or dead cells (67). Additionally, keratins from trout have been shown to display antibacterial activity (68) and these proteins will likely indicate danger to surrounding conspecifics. In addition to keratins, binding proteins were highly expressed in the current water samples from infected R line fish. Binding proteins, identified in the mucosa of fish have been shown to serve many functions including immune response and lipid metabolism (69, 70) and it is plausible that these proteins from the mucus are being detected in

current samples. Importantly, fish mucus has been shown to contain a plethora of immune related proteins including those from the complement (71), immunoglobulins (72) and antimicrobial peptides (73). Both NCBI and transcriptome based databases indicated immune related proteins in current fish water samples; these were identified for individual characterized proteins (e.g. cytokines) but also at the level of go annotation and pathway analyses whereby immune annotations and pathways were highly enriched. One current example of an immune protein identified by MASCOT and go annotation scoring, from infected R line individuals, is interleukin 6 signal transducer (IL6st). Interleukin 6 receptor was previously identified in the mucus of gilthead sea bream (*Sparus aurata*) that were chronically stressed (74) and several other interleukin molecules with well-known roles in inflammatory response and protection from pathogens have been described in the skin of Nile tilapia (*Oreochromis niloticus*) (75, 76). As fish mucus is continually secreted and replaced (75) it is likely that immune molecules, such as IL6, are transferred to the media on a constant basis. As demonstrated in the current study, the regeneration and movement of mucosal molecules into the aquatic media allows for collection and analysis of these proteins in low water volumes.

Further to specific immune or structural proteins, the fish mucosa of chronically stressed has allowed for research into a variety of stress related proteins thought to influence the response of fish to environmental fluctuations (67, 74). Proteins associated with oxidative stress response are commonly studied in fish exposed to toxic environmental pollutants, for example metals (77) and associated biomarkers have been suggested as a potential target for environmental monitoring and pollutant detection (78, 79). Additionally, these proteins have been identified in fish skin mucus and have roles in regulation of cell death (74, 80). Here we provide evidence that the OSER1 protein, of fish origin, was detectable from all samples of aquaria water and has the potential indicate environmental stress response in this species. In this instance OSER1 could be linked with stress related to experimental testing such as sham

infection (for control fish) or louse attachment for infected individuals. This warrants further investigation but confirms the ability of current methods to detect a well-known marker.

Many more proteins of interest appeared in two or three individual fish below the MASCOT cut-off. Presence of these proteins might have increased with a larger number of samples at a higher concentration and/or larger volumes of water. Briefly, infected fish samples and *Argulus* only samples contained proteins (below the MASCOT significance cut off) from mite and lice species; these included PHUM400690 (for immune response to ticks), capicua protein (a transcriptional repressor) and IscW (an immune suppressor). As only one parasite and a small host were tested in the current study it is likely that larger fish, with several parasites would reveal more clues about these proteins. If these proteins were discovered to increase with parasite number they would likely be a strong target for environmental protein detection methods. Proteins with roles in oxidative stress response, response to lice, pigmentation and immune response identified here have the potential to reveal infection and/or stress status in fish Proteins associated with *Argulus foliaceus* were observed in water from infected fish, yet several proteins were only identified in the *Argulus* control group. These proteins (zinc finger protein and proteins involved in the immune response to lice and ticks) demonstrated the possibility of using proteomic techniques to detect the presence of parasitic organisms within the environment, as with environmental DNA (81-83) . Lastly proteins with roles in oxidative stress response, response to lice, pigmentation and immune response identified here have the potential to indicate infection and/or stress status in fish, an area that requires further investigation. We propose that the proteomic methods outlined here have the potential to provide a similar tool for evaluating stress and health status in individuals, with the added benefit of functional analysis of expressed proteins. Lastly, we suggest that proteomic and environmental DNA methods could be used in tandem to gain non-invasive data on farmed and wild fish populations.

5. Concluding remarks

This is the first study outlining protein characterisation from brackish water samples without the need for gel electrophoresis and represents a major step forward in the field of metaproteomic techniques. Our novel assay provides a non-invasive method for detecting infection status in fish, which will be useful for assessing the health condition of both wild and farmed aquatic organisms. With further investigation and optimisation, protein detection methods represent a promising new method for environmental monitoring and could become an important tool in applied and aquatic biology.

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Author's contributions

RJP, SC & RS designed the study with help from PMB and RMM. RJP, SC & RMM wrote the manuscript. PMB provided the lab for experimental procedures. JC provided expertise and facilities for all experimental infections. CGL and JC provided manuscript corrections and advise.

Conflict of interest statement

The authors have declared no conflict of interest

Ethics approval

All the experiments in this study have been conducted following Home Office regulations, approved by both Swansea and Cardiff University Ethics Committees and under Home Office licence number PPL 302357.

SUPPORTING INFORMATION

Supplementary material_Proteomics.docx

1. **Details S1**. Extra details for methods and materials – sample processing, 1D SDS page, TCA methods.
2. **Table S1.** Protein number, percentage above mascot cut off, unique proteins and unidentified proteins from all groups and all fish replicates from both Transcriptome and NCBI data sets.
3. **Table S2**. Proteins identified on or equal to mascot score cut off, proteins found in all replicates and proteins for in two replicates from data obtain from the Mangrove Killifish Transcriptome
4. **Table S3.** Proteins identified on or equal to mascot score cut off, proteins found in all replicates and proteins for in two replicates from data obtain from the NCBI database
5. **Table S4.** Proteins of interest identified from Mangrove killifish transcriptome data and the NCBI database
6. **Table S5.** REACTOME pathway analysis for 5 groups of water samples from *Kryptolebias marmoratus*
7. **Figure S1:** Go annotations from transcriptome data (Level Go All) for all 5 groups of *Kryptolebias.marmoratus* (Dan infected, Dan control, R infected, R control and *Argulus* only) and all three replicates per group.
8. **Figure S2**: Go annotations from NCBI data (Level Go All) for all 5 groups of *Kryptolebias marmoratus* (Dan infected, Dan control, R infected, R control and *Argulus* only) and all three replicates from each group.
9. **Figure S3**. Pathway analysis using REACTOME for 3 biological replicates of *Kryptolebias marmoratus* water samples from 5 groups.

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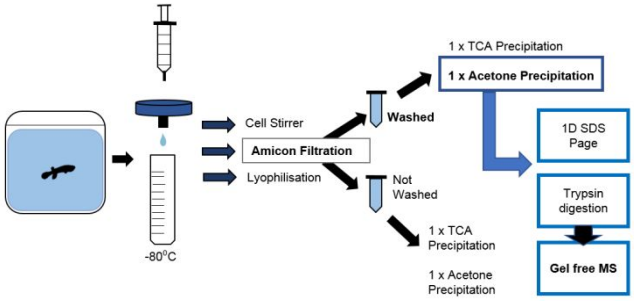
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