Proteomic identification of galectin-11 and 14 ligands from *Haemonchus contortus*

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Haemonchus contortus is the most pathogenic nematode of small ruminants. Infection in sheep and goats results in anaemia that decreases animal productivity and can ultimately cause death. The involvement of ruminant-specific galectin-11 (LGALS-11) and galectin-14 (LGALS-14) has been postulated to play important roles in protective immune responses against parasitic infection; however, their ligands are unknown. In the current study, LGALS-11 and LGALS-14 ligands in *H. contortus* were identified from larval (L4) and adult parasitic stages extracts using immobilised LGALS-11 and LGALS-14 affinity column chromatography and mass spectrometry. Both LGALS-11 and LGALS-14 bound more putative protein targets in the adult stage of *H. contortus* (43 proteins) when compared to the larval stage (2 proteins). Of the 43 proteins identified in the adult stage, 34 and 35 proteins were bound by LGALS-11 and LGALS-14, respectively, with 26 proteins binding to both galectins. Interestingly, hematophagous stage-specific sperm-coating protein and zinc metalloprotease (M13), which are known vaccine candidates, were identified as putative ligands of both LGALS-11 and LGALS-14. The identification of glycoproteins of *H. contortus* by LGALS-11 and LGALS-14 provide new insights into host-parasite interactions and the potential for developing new interventions.

1   **Proteomic identification of galectin-11 and 14 ligands from**

2   ***Haemonchus contortus***

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Abstract

Haemonchus contortus is the most pathogenic nematode of small ruminants. Infection in sheep and goats results in anaemia that decreases animal productivity and can ultimately cause death. The involvement of ruminant-specific galectin-11 (LGALS-11) and galectin-14 (LGALS-14) has been postulated to play important roles in protective immune responses against parasitic infection; however, their ligands are unknown. In the current study, LGALS-11 and LGALS-14 ligands in *H. contortus* were identified from larval (L4) and adult parasitic stages extracts using immobilised LGALS-11 and LGALS-14 affinity column chromatography and mass spectrometry. Both LGALS-11 and LGALS-14 bound more putative protein targets in the adult stage of *H. contortus* (43 proteins) when compared to the larval stage (2 proteins). Of the 43 proteins identified in the adult stage, 34 and 35 proteins were bound by LGALS-11 and LGALS-14, respectively, with 26 proteins binding to both galectins. Interestingly, hematophagous stage-specific sperm-coating protein and zinc metalloprotease (M13), which are known vaccine candidates, were identified as putative ligands of both LGALS-11 and LGALS-14. The identification of glycoproteins of *H. contortus* by LGALS-11 and LGALS-14 provide new insights into host-parasite interactions and the potential for developing new interventions.

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Introduction

65 *Haemonchus contortus* is a dominant blood feeding gastrointestinal nematode (GIN) parasite of

66 small ruminants. Blood feeding by *H. contortus* results in haemorrhagic gastritis, oedema,

67 diarrhoea and death in severe infections, leading to significant economic impact through

68 decreased livestock production (Mavrot et al. 2015; McLeod 1995; Roeber et al. 2013). Sheep

69 can develop effective immunity to *H. contortus* infection and vaccine-induced protection using

70 *H. contortus*-derived molecules such as the H-gal-GP, a gut-derived, galactose-containing

71 glycoprotein complex and integral membrane glycoprotein complex, termed H11, a family of

72 microsomal aminopeptidases have been demonstrated, suggesting that the control of this parasite

73 through vaccination is possible (Nisbet et al. 2016). However what host molecules recognise

74 these glycoproteins are poorly understood. Recently it has been shown that galectins have been

75 showed to play major roles in host defence against microbial pathogens. Galectins are a family of

76 carbohydrate-binding molecules with characteristic domain organization and affinity for β-

77 galactosides that mediates a variety of important cellular functions, including inflammation and

78 immune responses due to binding both self and non-self glycans (Vasta et al. 2017).

79 In particular, ruminants highly upregulate the expression of two specific galectins (LGALS-11

80 and LGALS-14) upon infection by various parasites such as *Ostertagia ostertagi, Cooperia*

81 *oncophora* and *H. contortus* (Dunphy et al. 2000; Dunphy et al. 2002; Hoorens et al. 2011;

82 Meeusen et al. 2005). LGALS-14 is secreted by eosinophil immune cells that are critical for

83 immunity through killing the larval stages of *H. contortus* (Balic et al. 2006; Dunphy et al. 2002;

84 Young et al. 2009). LGALS-14 is thought to be the homologue of human galectin-10, which is

85 also secreted by eosinophils (Ackerman et al. 2002). Analysis of *H. contortus* infected sheep

86 demonstrated release of LGALS-14 into the gastrointestinal mucus, the interface of host and

87 parasite interaction (Dunphy et al. 2002). In addition, kinetic studies of LGAL-14 showed that

88 release into the mucus occurred soon after challenge infection, and correlated with a reduction in

89   parasitic burden (Robinson et al. 2011). Additional it has been shown that LGAL-14 can bind

90   directly to another parasite *Fasciola hepatica* suggesting it can inhibit infection (Young et al.

91   2012).

92   The second galectin (LGALS-11) is specifically expressed and secreted during *H. contortus*

93   infections in previously infected sheep that had developed resistance to the parasite (Dunphy et

94   al. 2000). Immunohistochemistry revealed that LGALS-11 was secreted by epithelial cells lining

95   the gastrointestinal tract, where it was localised to the nucleus and cytoplasm of cells. Analysis

96   of the mucosal contents lining the gastrointestinal tract also revealed secretion of LGALS-11 into

97   the mucus. An observation of increased mucus stickiness corresponding with the production of

98   LGALS-11 suggested that it might work by interacting with the mucus to impede *H. contortus*

99   motility (Robinson et al. 2011). Recent immunofluorescent staining techniques using a

100   recombinant form of galectin-11 have revealed binding to the fourth larval stage and adult *H.*

101   *contortus* that has resulted in impaired development. These studies suggest a more direct or

102   additional role for LGALS-11 during *H. contortus* infections.

103   It appears that both LGALS-11 and LGALS-14 mediate critical immune regulatory effects

104   and/or mediate direct parasite stage-specific killing (Haslam et al. 1998; Preston et al. 2015b).

105   Although the interactions of these host galectin-parasite glycoconjugates are likely to be critical

106   for parasite control, the parasite glycoconjugate molecules that they recognise are unknown. For

107   the first time, this study describes the ligands of sheep LGALS-11 and LGALS-14 in larval and

108   adult stages of *H. contortus*.

109

110   # Materials and Methods

111   ## Preparation of L4 larvae and collection of adult parasites

112   *H. contortus* (Haecon-5 strain) was maintained in Professor Gasser's laboratory, Melbourne

113   Veterinary School, The University of Melbourne and was used in this study. Mature fourth stage

114   larvae (L4 stage) and adults of *H. contortus* were prepared using established protocols (Preston

115   et al. 2015a). Briefly, third-stage larvae (L3) were isolated from faeces from *H. contortus*-

116   infected sheep. The cuticle was removed from the L3s by using sodium hypochlorite, the

117   exsheathed L3 (xL3) worms were washed three times with 0.9% (w/v) normal saline.

118 Approximately 2000 xL3 / ml worms were resuspended in Dulbecco's modified Eagle

119 Medium+GlutaMax (DMEM) (Gibco-Invitrogen, USA) containing 10,000 IU/ml of penicillin

120 and 10,000 μg/ml of streptomycin (Gibco-Invitrogen, USA) and 0.5 % (v/v) amphotericin (GE

121 Healthcare, UK). Medium containing xL3s was incubated at 37 °C with 10 % (v/v) CO_2 for 7

122 days. Fresh DMEM was substituted at two-day intervals and larval development was examined

123 each day. The xL3 and L4 stages were differentiated based on distinctive morphological

124 characteristics (see Preston et al., 2015a). Animal experimental procedures were approved by the

125 Monash University Animal Ethics Committee (Ethics # SOBSA/P/2009/44). Adults of *H.*

126 *contortus* were collected from Merino ewes (8-12 months old) which were experimentally

127 infected with 10,000 L3s and the infected animals were euthanised 52 days post infection by

128 injection of pentobarbitone (Lethobarb®, Virbac Pty Ltd, Australia). Approximately 5,000 adult

129 worms of mixed sex were collected from the abomasal content and washed five times with 0.9 %

130 (v/v) biological saline (Baxter, Australia). Immediately after washing, the worms were snap

131 frozen in liquid nitrogen and stored at -80 °C until further use.

132 **Total larval protein lysate preparation**

133 Lysates were prepared using radioimmunoprecipitation assay buffer (RIPA) as previously

134 described with minor modifications (Maduzia et al. 2011). Briefly, 500 mg of larval or adult *H.*

135 *contortus* were incubated with 100 mM β-D-galactose containing 0.9 % (v/v) normal saline for

136 12 h to remove native galectins (bound to the adult parasite surface recovered from infected

137 sheep) and washed three times with normal saline. Larval or adult *H. contortus* were then

138 resuspended in 5 ml of ice-cold RIPA buffer [20 mM Tris-HCL pH 7.2, 100 mM NaCl, 1% (v/v)

139 Nonidet P-40, 0.1 % (w/v) sodium deoxycholate (DOC), 0.05 % (w/v) sodium dodecyl sulphate

140 (SDS), 1 % (v/v) Triton X-100, 10 mM TCEP (Tris (2-carboxyethyl) phosphine)] and lysed by

141 sonication (30 sec, 8 times with three min interval at 40 % amplitude). Cellular debris was

142 removed by centrifugation (15000 x *g* for 20 min) at 4 °C, and any particles in the supernatant

143 removed by filtering through a 0.22 μm filter. Lysates were dialysed using 3 kDa molecular

144 weight cut-off against binding buffer [(20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5 % (v/v)

145 Nonidet P-40, 0.1 % (w/v) DOC, 0.05 % (w/v) SDS, 1% (v/v) Triton X-100, 10 mM TCEP)].

146
147 SDS–PAGE

148 **LGALS-11 and LGALS-14 affinity column**

149 Recombinant LGALS-11 and LGALS-14 were expressed and purified as described previously

150 ((Sakthivel et al. 2015); Fig. 1). The recombinant protein (5 mg/ml) was buffer-exchanged into

151 HEPES buffer (10 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 10 mM TCEP) and immobilised

152 by coupling to N-hydroxysuccinamide (NHS)-activated Sepharose (GE Healthcare, UK)

153 following the manufacturer's protocol. Briefly, 4 ml of NHS-activated Sepharose was washed

154 with 15 column-volumes of ice-cold 1 mM HCl. The washed Sepharose beads were equilibrated

155 with 20 ml of coupling buffer (10 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 10 mM TCEP).

156 Following equilibration, LGALS-11 and LGALS-14 were added separately to the activated

157 Sepharose and allowed to couple for 5 h at 22 °C. Following the coupling reaction, the unused,

158 activated sites were blocked using 15 column-volumes of blocking buffer (100 mM Tris-HCl pH

159 8.0, 100 mM NaCl) for 3 h. Following blocking, the Sepharose beads were washed alternatively

160 six times with 15 column-volumes of 100 mM Tris-HCL pH 8.0 followed by 100 mM sodium

161 acetate pH 5.0 and then 250 mM NaCl. The galectin affinity column was maintained in storage

162 buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM TCEP, NaAc 0.02 % (w/v)) until further

163 use. A control resin was also prepared without any protein ligand.

164

Isolation of LGALS-11 and LGALS-14 parasite ligands

166 Immobilised LGALS-11, -14 or control slurry (1 ml) was loaded into individual columns. Larval

167 and adult *H. contortus* lysates (150 mg) were diluted with 5 ml of binding buffer (20 mM Tris-

168 HCl pH 7.5, 100 mM NaCl, 0.5 % (v/v) Nonidet P-40, 0.1 % (w/v) DOC, 0.05 % (w/v) SDS, 1%

169 (v/v) Triton X-100, 10 mM TCEP) and applied to the galectin affinity column and incubated for

170 16 h at 4°C. Thereafter, columns were washed three times with 15 ml of RIPA buffer, the

171 captured protein fractions were eluted by incubating for 2 h with galactose elution buffer (250

172 mM β-D-galactose 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM TCEP) and the resultant

173 supernatant was subjected to LC-MS/MS analysis to identify the protein molecules present. The

174 eluted protein products were analysed by 12% SDS-PAGE stained with nitrate. The unbound

175 fractions, column wash and eluted proteins fractions were concentrated using sodium

176 deoxycholate/trichloroacetic acid precipitation method to allow the visualisation of protein

177 products as previously described (Arnold & Ulbrich-Hofmann 1999).

178

179 **Mass spectrometry (ESI–LC–MS/MS) analysis of galectin binding proteins**

180   Eluted protein samples were dissolved in digestion buffer (8 M urea, 50 mM NH$_4$HCO$_3$, 10 mM

181   dithiothreitol) and incubated at 25 °C for 5 h. Following incubation, iodoacetamide (IAA) was

182   added to final concentration of 55 mM to alkylate thiol groups and incubated for 35 min at 20 °C

183   in the dark. The alkylated protein preparation was diluted with 1M urea in 25 mM ammonium

184   bicarbonate (pH 8.5) and sequencing-grade trypsin (Promega) was added to a final concentration

185   of 5 μM. The reaction was incubated for 16 h at 37 °C in the dark. The digests were acidified

186   with 1% (v/v) trifluoroacetic acid (TFA) and the peptides desalted on poly(styrene-

187   divinylbebzebe) copolymer (SDB) (Empore) StageTips as described previously (Rappsilber et al.

188   2007).

189   Trypsin-digested peptides were reconstituted in 0.1% (v/v) TFA and 2% (v/v) acetonitrile (ACN)

190   and then loaded onto a guard column (C$_{18}$ PepMap 100 μm ID × 2 cm trapping column, Thermo-

191   Fisher Scientific) at 5 μl/min and washed for 6 min before switching the guard column, in line

192   with the analytical column (Vydac MS C$_{18}$, 3 μm, 300 Å and 75 μm ID × 25 cm). The separation

193   of peptides was performed at 300 nl/min using a non-linear ACN gradient of buffer A (0.1%

194   (v/v) formic acid, 2 % (v/v) ACN) and buffer B (0.1% (v/v) formic acid, 80 % (v/v) ACN),

195   starting at 5% (v/v) buffer B to 55% for 120 min. Data were collected on an Orbitrap Elite

196   (Thermo-Fisher Scientific) in a data-dependent acquisition mode using m/z 300–1500 as MS

197   scan range, CID MS/MS spectra and were collected for the 20 most intense ions. Dynamic

198   exclusion parameters were set as described previously (Nguyen et al. 2016). The Orbitrap Elite

199   was operated in dual analyser mode, with the Orbitrap analyser being used for MS and the linear

200   trap being used for MS/MS. Pull-down and LC-MS/MS analysis were performed three times on

201   different days.

202

203 **Database search and protein identification**

204   The MS/MS spectra obtained from the Orbitrap analyser was used to interrogate the Swiss-Prot

205   *Haemonchus contortus* FASTA database (downloaded on 07.31.2016, 21,201 protein entries) in

206   conjugation with common contaminants by use of the Mascot search engine (Matrix Science

207   Ltd., London, UK) as described previously (Perkins et al. 1999). Briefly, carbamidomethylation

208   of cysteines was set as a fixed modification, acetylation of protein N-termini, methionine

209   oxidation were included as variable modifications. Precursor mass tolerance was 10 ppm,

210   product ions were searched at 0.5 Da tolerances, minimum peptide length defined at 6, maximum

211   peptide length 144, and peptide spectral matches (PSM) were validated using Percolator based

212   on q-values at a 1% false discovery rate (FDR). Both peptide and protein identifications were

213   reported at a false discovery rate (FDR) of 1%. The mass spectrometry proteomics data have

214   been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the

215   data set identifier PXD008435 and 10.6019/PXD008435.

216   **Protein-protein interaction analysis and visualisation**

217   Normalized spectral abundance factor (NSAF) values were calculated for the identified proteins

218   using the Scaffold software v4.7.2 (Searle 2010). Then proteins were subjected to the

219   significance analysis of interactome (SAINT) (Choi et al. 2011) to identify *bona fide* protein-

220   protein interactions after removing all zero or missing rows. Proteins with a SAINT probability

221   greater than 0.9 were selected as high probability interactions. Finally, the resulting interaction

222   network was visualised using the Cytoscape v3.4.0 (Shannon et al. 2003).

223
224   **Analysis of glycosylation**
225
226   The N- and O-linked glycosylation pattern and the signal peptides of eluted proteins were

227   analysed following the instructions provided in the glycosylation analysis server. Briefly, N-

228   glycosylation and signal peptide was analysed using NetNGlyc 1.0 server

229   (http://www.cbs.dtu.dk/services/NetNGlyc/). Whereas the O-glycosylation pattern was analysed

230   using NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/). The results obtained

231   from the N and O glycosylation servers were provided as supplementary results.

232

233   # Results and Discussion
234
235   **Identification of proteins from *H. contortus* that interact with LGALS-11 and LGALS-14**

236   Affinity purification and identification of *H. contortus* proteins interacting with LGALS-11 and

237   LGALS-14 is summarized in Fig. S1. Lysates of L4 and adult stages were assessed before

238   loading onto the columns containing the Sepharose immobilised LGALS-11 and LGALS-14 and

239   are shown in Fig. 1a. Multiple bands were observed, with both larval and adult lysates containing

240   a broad range of proteins of differing molecular weights. Following the application of L4 and

241   adult lysates to affinity columns, containing immobilised galectins, bound parasite molecules

242   were eluted with elution buffer-containing galactose (Fig. 1b & 1c). The eluted molecules from

243   both affinity columns and a control column were subjected to LC-MS/MS. Proteins that were

244   identified in 2 of the 3 biological replicates were included for further analysis and the proteins

245   that were bound to control resin (Table S1) were removed from the analysis. Overall, 43

246   individual proteins were identified and grouped based on their respective known or putative

247   biological function(s) (Table 1). The greatest number of proteins identified was in the adult stage

248   of *H. contortus*; with 34 proteins binding to LGALS-11 and 35 proteins binding to LGALS-14.

249   Of those identified proteins, 26 proteins were found to bind to both LGALS-11 and LGALS-14

250   (Fig. 2). In the L4 larval stage, LGALS-11 and LGALS-14 could bind to 0 and 2 proteins

251   respectively.

252

253   **Composition of LGALS-11 and LGALS-14 ligands**

254   Approximately 69% of proteins in *H. contortus* that bound specifically to LGALS-11 and/or

255   LGALS-14 were inferred to be involved in metabolic and regulatory processes (Table 1, Fig. 3).

256   Most of these proteins (~ 70 %) were likely involved in metabolic activities, such as energy

257   metabolism, transcription and translation. These proteins predominantly included regulatory

258   enzymes, such as peptidases, carboxyl transferases, aldo-keto reductases, deoxynucleoside

259   kinase, dehydrogenase, amidinotransferase and RNA polymerase. Another protein group (~9 %)

260   identified represented structural proteins, such as actin, myosin and collagen from *H. contortus*

261   (Table 1, Fig. 3). Other proteins identified had putative roles in molecular transport (e.g., lipid

262   and amino acid transport) or had no assigned function(s) (Fig. 3). *In silico* analysis revealed that

263   approximately 65% of the proteins of the adult stage, that bound specifically to LGALS-11 and

264   LGALS-14 had one or more potential glycosylation site (Table 2). On the contrary, about 35 %

265   of adult stages specific proteins that bound to LGALS-11 and LGALS-14 were predicted as non-

266   glycosylated. Though animal lectins have a primary preference for glycoconjugates, it is believed

267   that the LGALS-11 and LGALS-14 might also display a glycan independent protein-protein

268   interaction activity similar to previously reported for galectin-1 and galectin-3 (Bawumia et al.

269   2003; Camby et al. 2006; Menon et al. 2000; Paz 2001). Galectin-3 has also been shown to

270   interact with proteoglycans which are proteins which have covalently attached

271 glycosaminoglycan chain (Talaga et al. 2016). Most mammalian proteoglycans are major

272 component of extracellular matrix such collagen. A nematode cuticle collagen and collagen triple

273 helix repeat protein (W6NAH7) was identified in adult stages by LGALS-11 and LGALS-14

274 suggesting that LGALS-11 and LGALS-14 can interact with proteoglycans.

275

276 **Larval and adult ligands of LGALS-11 and LGALS-14**

277 More putative ligands (n = 43) were identified in the adult stage of *H. contortus* compared with

278 larval stages (n = 2) following galectin pull-down assays. Although the L4 stage is a histotropic

279 stage (in glands of the stomach) and would be expected to be in intimate contact with

280 inflammatory mediators, including galectins, it moults (with a change in antigenic profile) within

281 48-72 h into the immature adult (Meeusen et al. 2005). This would be expected to limit the

282 antigenic exposure of these parasite antigens to the host. Compared to the adult stage that is

283 relatively long-lived (6-8 weeks), allowing a sustained interaction of host molecules with

284 parasite antigens (Nikolaou & Gasser 2006; Veglia 1915). This interaction might be reflected in

285 the specific and localised binding of LGALS-11 in the larvae and the significant staining of

286 LGALS-11 on the surface of adult *H. contortus* (see Preston et al., 2015b). In addition, the L4

287 stage is relatively small (750 – 850 μm long), whereas the adult stage is usually 10-30 mm long.

288

289 A protein-protein interaction network was drawn for LGALS-11 and LGALS-14 affinity purified

290 proteins specific to adult parasitic stage revealed that, LGALS-11 and LGALS-14 found to

291 interact 5 unique proteins individually. Whereas 9 proteins were found to interact with both

292 LGALS-11 and LGALS-14 (Fig. 4). Carboxyl transferase, aldo keto reductase and myosin

293 displayed unique interaction with LGALS-11. Whereas zinc metallopeptidase M13, porin

294 domain containing protein, von Willebrand factor-like domain containing protein and

295 mitochondrial solution substrate carrier protein displayed an interaction network unique to

296 LGALS-14. Peptidase S28, alpha beta hydrolase fold-1, glutamate phenylalanine leucine valine

297 dehydrogenase, nematode cuticle collagen, lipid transport protein containing vitellinogen and

298 von Willebrand factor-like domains were found to interact both LGALS-11 and LGALS-14

299 (Fig.4).

300 **Protease and phosphatase ligands**

301 A significant number of proteins (n = 19) with enzyme activity were identified in adults, and

302 similar proteins have been described in other 'omic' studies, suggesting that many of these

303 enzymes of the protease family are conserved and evolutionarily related in nematodes (Campbell

304 et al. 2011; Ghedin et al. 2007; Schwarz et al. 2013). A notable protease identified in the adult

305 stage, is zinc metallopeptidase (M13 protease or neprilysin). Zinc metallopeptidases have been

306 reported as the major protein fraction of host protective glycoprotein complex H-gal-GP

307 (*Haemonchus* galactose containing glycoprotein). Another important metalloprotease is H11

308 family which has been shown to provide protection when used as vaccine antigen (Newton &

309 Munn 1999). Several studies isolated zinc metallopeptidases from crude extracts of *H. contortus*

310 using lectins that have a binding preference to β-D-galactose and, following vaccination of

311 sheep, led to reduced worm burdens following challenge infection (Dicker et al. 2014; Newlands

312 GFJ 2006; Smith et al. 1999; Smith et al. 2000). It appears inhibition of metalloproteases

313 interferes with ability of *H. contortus* to digest its blood meal.

314

315 Another important group of proteases were found to interact with LGALS-11 and LGALS-14,

316 which are S28 family (Table 1). Four different S28 proteases interacted were shown to interact

317 with LGALS-11 and LGALS-14 in particular HCOI_01497800, HCOI_01562400 and

318 HCOI_01624000 which have homology to contortin (Fig. S2). Contortin is intestinal antigen of

319 *H. contortus* which induced significant levels of protection when used to vaccinate lambs (Munn

320 et al. 1987). Contortin is comprised of two proteins, Hc-PCP1 and Hc-PCP2 that can degraded

321 the C-terminal end of the fibrinogen alpha-chain therefore inhibiting blood clotting (Geldhof &

322 Knox 2008).

323

324 **Blood ligands**

325 A number of parasite molecules were identified that interact with host galectins and are

326 potentially involved in manipulating the host blood function in the adult stage but not larvae of

327 *H. contortus*. That the adult stage of this nematode is primary a blood feeder may explain the

328 lack of such molecules identified in the larvae. Blood feeding parasites are known to use several

329 mechanisms to suppress platelet aggregation, allowing prolonged blood feeding by retarding

330 blood clotting (Liu & Weller 1992). The von-Willebrand factor (VWF) is a well-known protein

331   reported in integrin and other extracellular proteins (Whittaker & Hynes 2002). The binding of a

332   C-type lectin (CLEC4M), with VWF has previously been shown to enhance the internalisation of

333   VWF by the host cells and alter plasma levels of VWF (Rydz et al. 2013). In previous reports,

334   proteins containing the VWF-like domain are localised in nematode intestine and suggested to

335   play critical roles in cell adhesion and platelet aggregation (Wohner et al. 2012). A multimeric

336   glycoprotein containing VWF-like domain was identified previously in adult *H. contortus* that

337   can suppress platelet aggregation (Crab et al. 2002). In this study, a protein containing a VWF-

338   like domain (W6ND82) was eluted from the LGAL14 column, which might suggest that this

339   host galectin plays a role in potential modulating the ability of the parasite to suppress blood

340   clotting. This protein was not detected in larval stage by both LGALS-11 and LGALS-14.

341   However, the functional significance of VWF domain containing proteins in parasitised animals

342   remains unknown, warranting further study.

343

344   **Specific sperm-coating protein (SCP)**

345   The stage-specific sperm-coating protein (SCP) identified interacting with host galectins in this

346   study are common to many nematode species (Cantacessi & Gasser 2012) and are suggested to

347   play critical roles in infection and immunomodulatory events such as neutrophil inhibition

348   (Cantacessi et al. 2012; Gadahi et al. 2016; Hewitson et al. 2009). Transcriptomic studies of *H.*

349   *contortus* have identified that 54 genes containing one or more SCP-like domains are

350   upregulated in the blood-feeding adult, suggesting that SCP proteins have active and stage-

351   specific involvement at the onset of blood feeding (Wang & Kim 2003). Similar SCP domain

352   containing proteins (Hc24 and Hc40) were reported in excretory/secretory proteins of *H.*

353   *contortus* (Yatsuda et al. 2003). Although there is some information for SCP domain-containing

354   proteins in *C. elegans* (O'Rourke et al. 2006; Wang & Kim 2003), their biological functions in

355   *H. contortus* needs experimental investigation.

356

Conclusion

358   Recently, host galectins have been hypothesised to interact with molecules to modulate host-

359   pathogen interactions in ruminants (Hoorens et al. 2011; Kemp et al. 2009; Preston et al. 2015b).

360   The finding that LGAL-14 is concentrated within eosinophils (an immune cell considered a

361   major mediator of parasite killing, including of *H. contortus*) suggested the possibility of a direct

362   role for ruminant galectins in mediating parasite-killing (Meeusen & Balic 2000; Robinson et al.

363   2011). The subsequent demonstration of direct binding of LGAL-11 to *H. contortus* and their

364   ability to inhibit larval development and growth *in vitro* has confirmed the roles of galectins and

365   ability to directly kill relatively large multicellular pathogens (Preston et al. 2015b).

366   The parasite surface is the key contact with the host and is often considered important source of

367   potential vaccine molecules. Correspondingly, 45% of the glycoproteins that the two galectins

368   bound were membrane proteins of the adult stage of *H. contortus,* and included vitelline, myosin

369   and M13 protein (neprilysin); these proteins have been previously assessed as vaccine candidates

370   (Knox 2011; Strube et al. 2015; Tellam et al. 2002). This evidence would indicate that other

371   putative glycoproteins identified here by these ruminant galectins might facilitate the

372   identification of new intervention targets and, thus, warrant further investigation. In conclusion,

373   the analysis of parasite proteins recognised by galectins that are involved in resistance to

374   parasites (Guo et al. 2016; Preston et al. 2015a; Preston et al. 2015b), has identified several

375   interesting stage-specific proteins. Exploring the possible biological roles and potential

376   antihelminthic activities of these proteins has significant potential to advance our understanding

377   of the host-parasite interplay and inform future parasite control strategies.

378

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380

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383

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Identification by mass spectroscopy of larval and adult *Haemonchus contortus* proteins eluted from LGALS-11 and LGALS-14 columns

Accession code	Gene description	CV	PSMs	UP	Mascot Score	Groups				Log-odds	NSAF	MW (kDa)	Signal peptide	Number of N-and (blue) /or O-glycosylation sites (red)#
						1	2	3	4					
Metabolic process														
W6NE18	Peptidase S28 GN=HCOI_01497800	45.21	634	20	7058	No	Yes	Yes	Yes	145.70	0.071	67.1	No	1/5
W6NFG0	Alpha beta hydrolase fold-1 GN=HCOI_00457700	58.65	504	16	6681	No	Yes	Yes	Yes	128.68	0.129	36.6	Yes	1/0
W6NFT9	Peptidase S28 GN=HCOI_01562400	23.27	222	18	3151	No	Yes	No	Yes	11.26	0.0062	128.6	Yes	7/35
W6NJ96	Carboxyl transferase GN=HCOI_00766200	23.39	123	10	1146	No	Yes	No	Yes	16.01	0.0103	56.1	No	0/7
W6NLA8	Glutamate phenylalanine leucine valine dehydrogenase GN=HCOI_01838800	42.50	102	10	957	No	Yes	No	Yes	20.61	0.020	26.8	No	0/0
W6NG90	Peptidase S28 GN=HCOI_01624000	4.88	101	3	1218	No	Yes	No	Yes	29.6	0.003	117.0	Yes	4/7
W6NC58	Aldo keto reductase GN=HCOI_00043700	39.64	83	10	789	No	Yes	No	Yes	-0.18	0.009	38.0	No	3/6
W6NAV8	Aldo keto reductase GN=HCOI_00043500	27.22	46	6	477	No	Yes	No	Yes	-0.18	0.0028	40.0	No	2/4
W6NU27	Carboxyl transferase GN=HCOI_00766300	25.83	67	6	909	No	Yes	No	Yes	4.35	0.0023	42.2	No	1/0
W6NKM1	Succinate dehydrogenase iron-sulfur subunit, GN=HCOI_01735500	32.61	64	8	521	No	Yes	No	Yes	34.83	0.0067	31.6	No	1/10
W6NF70	Deoxynucleoside kinase GN=HCOI_01673500	35.42	42	6	242	No	No	No	Yes	-0.18	0.0012	34.8	No	0/0
U6NNG6	Ribosomal protein L7 L12 GN=HCOI_00340500	11.41	30	1	783	No	Yes	No	Yes	-0.18	0.013	19.5	No	1/3
W6NA79	Zinc metallopeptidase M13 GN=HCOI_01030800	37.90	28	3	330	No	No	No	Yes	4.35	0.0067	14.6	No	0/2
W6ND82	von Willebrand factor domain containing protein GN=HCOI_01354500	13.82	26	3	371	No	No	No	Yes	16.01	0.0077	27.5	Yes	2/0
W6NMI7	Proteinase inhibitor I33 GN=HCOI_02015200	18.58	22	5	162	No	No	No	Yes	-0.18	0.0020	25.1	Yes	0/7
W6NKG5	Ribosomal protein L15 GN=HCOI_01717500	5.88	4	1	26	No	No	No	Yes	5.67	0.0002	24.3	No	1/10
W6NEW9	Amidinotransferase GN=HCOI_01556200	23.35	24	4	109	No	Yes	No	No	-0.18	0.0014	29.2	No	0/0
W6NI22	Adenylosuccinate lyase GN=HCOI_00436500	6.41	14	3	141	No	Yes	No	No	12.23	0.0005	76.9	No	1/4

ID	Description													
W6NF84	Short-chain dehydrogenase reductase GN=HCOI_01467500	3.07	11	1	137	No	Yes	No	Yes	1.71	0.0003	27.5	No	0/0
W6ND43	Acetyltransferase component of pyruvate dehydrogenase complex GN=HCOI_00576100	1.22	8	1	51	No	Yes	No	No	1.68	0.0001	78.3	No	1/40
Regulation of biological processes														
W6NC73	ATPase GN=HCOI_02138200	22.64	23	2	418	No	Yes	No	Yes	-0.18	0.004	17.8	No	1/0
W6NJ12	Filament domain containing protein GN=HCOI_02013700	14.09	20	3	291	No	Yes	No	Yes	-0.18	0.0028	34.2	No	0/4
W6NM02	Fumarate lyase GN=HCOI_01914600	4.73	8	1	62	No	Yes	No	No	4.61	0.0003	29.7	No	1/12
W6NGK5	CRE-DHS-15 protein GN=HCOI_00341400	29.31	6	1	210	No	No	No	Yes	-0.18	0.0031	6.1	No	0/1
W6NI80	Acyl-CoA-binding protein GN=HCOI_01539100	20.69	6	1	205	No	Yes	No	Yes	6.69	0.0018	9.5	No	0/1
W6NCC1	NIPSNAP GN=HCOI_01963300	7.63	6	1	75	No	Yes	No	No	-0.18	0.0013	26.5	No	0/6
W6NWY9	Porin domain containing protein GN=HCOI_01573900	43.97	82	9	1149	No	Yes	No	Yes	4.35	0.011	30.2	No	3/0
W6NAW4	FG-GAP and Integrin alpha-2/Integrin alpha chain GN=HCOI_01903100	5.90	33	5	340	No	Yes	No	Yes	-0.18	0.0028	133.9	Yes	5/18
W6NAL4	Heat shock protein 70 GN=HCOI_00589700	7.24	9	1	82	No	Yes	No	No	7.23	0.0002	72.4	No	3/7
Transport														
W6NVQ1	Lipid transport protein and Vitellinogen and von Willebrand factor domain GN=HCOI_01683400	23.18	209	24	1465	No	Yes	No	Yes	9.62	0.0039	156.0	No	0/11
W6N9I2	Lipid transport protein GN=HCOI_00072100	35.97	139	13	1313	No	Yes	No	Yes	51.51	0.0136	56.8	Yes	0/2
W6NQZ5	Mitochondrial substrate solute carrier GN=HCOI_01092000	23.10	53	8	334	No	No	No	Yes	6.2	0.0033	36.3	No	0/3
Cytoskeleton														
W6NAH7	Nematode cuticle collagen and Collagen triple helix repeat GN=HCOI_00810700	5.60	85	2	1399	No	Yes	No	Yes	4.35	0.0117	32.8	No	0/15
W6NHH0	Annexin GN=HCOI_01003500	14.01	22	3	135	No	Yes	No	No	-0.18	0.0008	29.4	No	0/1
W6NE41	Myosin tail GN=HCOI_01216000	3.99	17	3	59	No	Yes	No	No	13.42	0.0008	54.7	No	0/15
W6NF56	Myosin tail GN=HCOI_01461300	34.48	51	8	675	No	Yes	No	Yes	6.2	0.0040	36.5	No	0/14

Immunomodulatory														
W6NGA7	SCP extracellular domain GN=HCOI_01577700	14.95	4	2	59	No	Yes	No	Yes	-0.18	0.0011	22.2	No	1/1
Unknown														
W6NAN9	CBN-MLC-3 protein GN=HCOI_01274700	49.67	63	7	465	No	Yes	No	Yes	2.32	0.0086	17.0	No	0/2
W6NX42	Uncharacterized protein GN=HCOI_01051700	25.56	38	3	607	No	No	No	Yes	19.09	0.0066	25.5	No	4/40
W6NFI4	Protein C15F1.2 GN=HCOI_01126300	34.87	20	4	97	No	Yes	No	Yes	-0.18	0.0022	27.1	Yes	0/13
W6NB42	Protein C23H5.8, isoform-c GN=HCOI_00648100	14.43	16	2	170	No	Yes	No	Yes	10.80	0.0025	22.5	Yes	1/1
W6NPK3	Uncharacterized protein GN=HCOI_00260500	5.20	10	1	45	No	No	No	Yes	-0.18	0.0001	22.6	Yes	3/11
W6NUX4	Uncharacterized protein GN=HCOI_01608400	4.04	12	1	43	No	Yes	No	Yes	4.04	0.0002	21.8	Yes	0/6

Abbreviations:

GN = Gene name; CV = Coverage; PSMs = Peptide spectrum matches; UP = Unique peptides;

Log-odds 0 = 50% chance, Positive values = More than 50%, Negative values = Less than 50%

Groups:

1 = LGALS-11 bound protein from L4 larval stage of *H. contortus*

2 = LGALS-11 bound protein from adult stage of *H. contortus*

3 = LGALS-14 bound protein from L4 larval stage of *H. contortus*

4 = LGALS-14 bound protein from adult stage of *H. contortus*

[#]Predicated glycosylation sites as determined in materials and methods.

NSAF = Normalized Spectral Abundance Factor

Figure 1

LGALS-11 and LGALS-14 interactome

(A) Protein profiling of larval and adult stages of _Haemonchus contortus_. (M)
Molecular weight markers; (Lane 1 & 2) lysates prepared from L4 stage; (Lane 3) lysates
prepared from adult stage**. (B) Protein profile of adult stage parasite bound to LGALS-
11 and LGALS-14 and (C) larval stage parasite bound to LGALS-11 and LGALS-14.**
M) Molecular weight markers; (Lane 1) Total parasite lysate, (Lane 2 & 3) Unbound protein
fractions of LGALS-11 and LGALS-14 column, (Lane 4 & 5) Column wash of LGALS-11 and
LGALS-14 column and (Lane 6 & 7) eluted protein of LGALS-11 and LGALS-14 column.

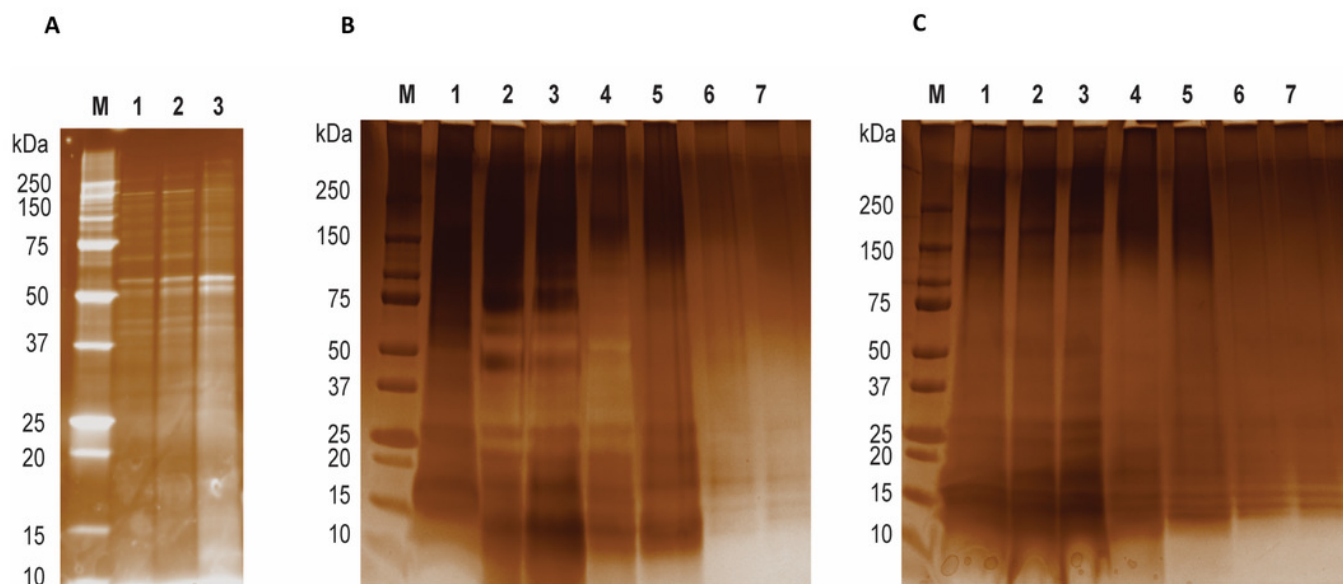



Figure 2

Venn diagram of parasite proteins bound by host galectins.

Venn diagram showing the distribution of proteins of the larval (A) and adult (B) stages of *Haemonchus contortus*. In larval and adult stages, 0 and 26 proteins were bound by both the galectins respectively.

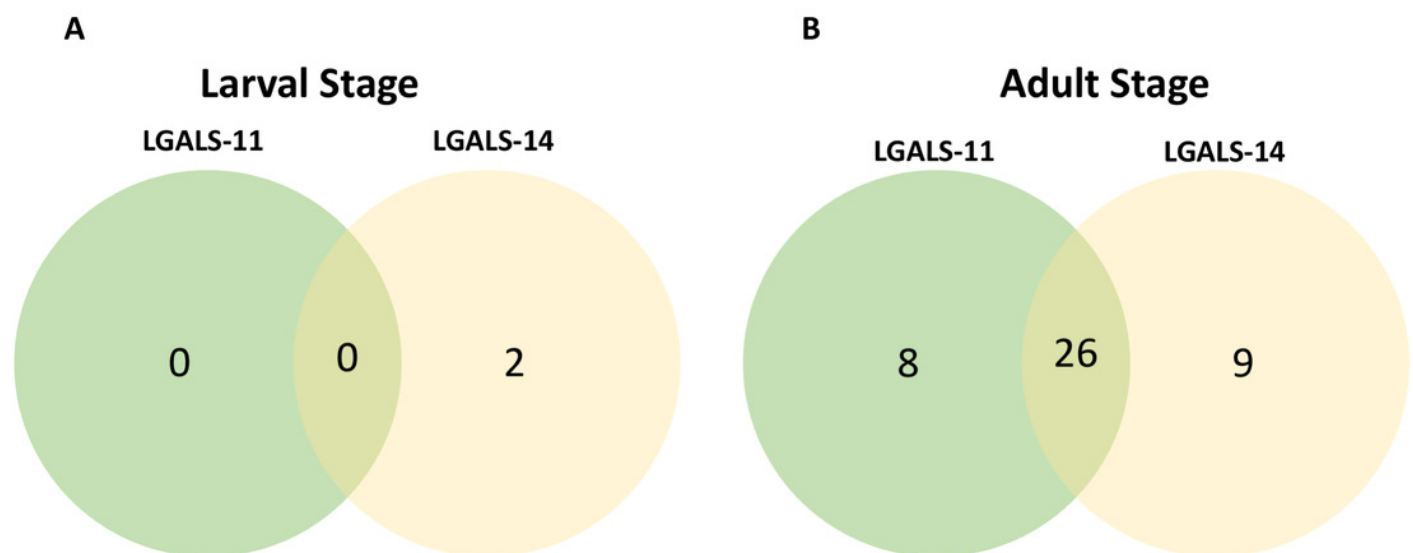
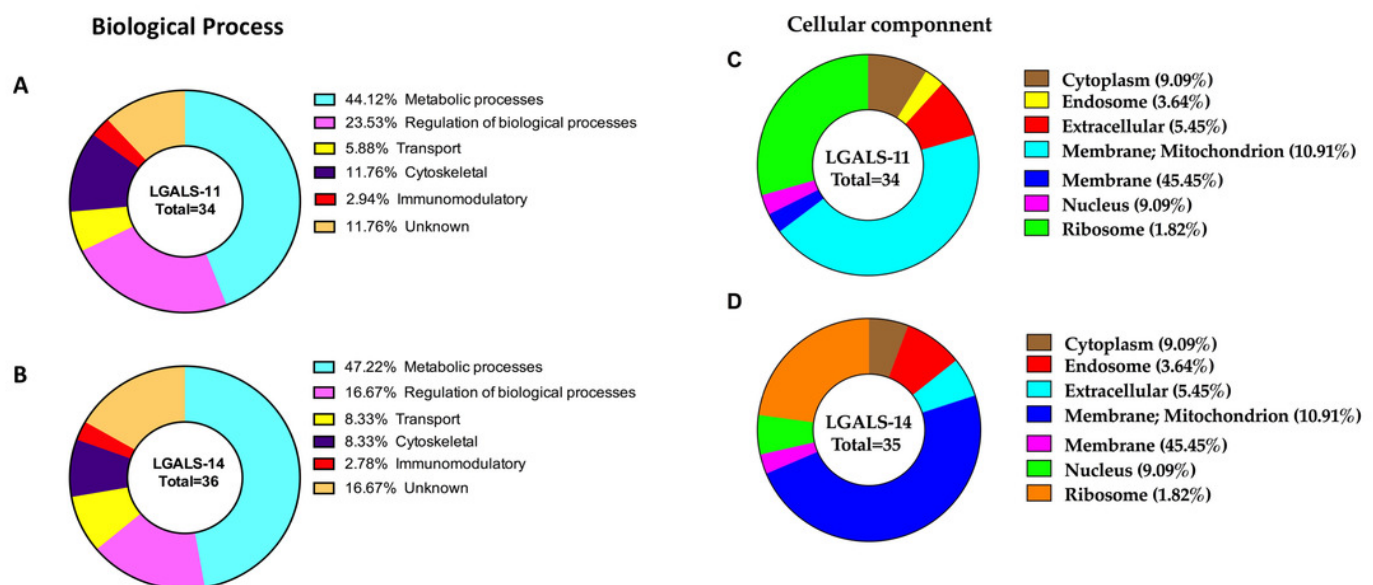



Figure 3

Categorisation of proteins in the adult stage of *Haemonchus contortus* that interacted with host galectins.

The profiles were categorised based on biological process of LGALS-11-bound proteins (A) and LGALS-14 bound-proteins (B) and cellular location of LGALS-11-bound proteins (C) and LGALS-14-bound proteins (D).

Protein-protein interaction network of adult stage of *Haemonchus contortus* with host galectins.

Protein interactions was determined using the software (SAINT) (Choi et al. 2011) and resulting interaction network was visualised using the Cytoscape v3.4.0.