# Characterisation of the secreted apyrase family of *Heligmosomoides polygyrus*

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**Abstract**

Apyrases are a recurrent feature of secretomes from numerous species of parasitic nematodes. Here we characterise the five apyrases secreted by *Heligmosomoides polygyrus*, a natural parasite of mice and a widely used laboratory model for intestinal nematode infection. All five enzymes are closely related to soluble calcium-activated nucleotidases described in a variety of organisms, and distinct from the CD39 family of ecto-nucleotidases. Expression is maximal in adult worms and restricted to adults and 4th stage larvae. Recombinant apyrases were produced and purified from *Pichia pastoris*. The five enzymes showed very similar biochemical properties, with strict calcium dependence and a broad substrate specificity, catalysing the hydrolysis of all nucleoside tri- and diphosphates, with no activity against nucleoside monophosphates. Natural infection of mice provoked very low antibodies to any enzyme, but immunisation with an apyrase cocktail showed partial protection to reinfection, with reduced egg output and parasite recovery. The most likely role for nematode secreted apyrases is hydrolysis of extracellular ATP, which acts as an alarmin for cellular release of IL-33 and initiation of type 2 immunity.

**Keywords:** Apyrase, nematode, helminth, *Heligmosomoides polygyrus*, vaccination

## 1. Introduction

Helminth parasites have evolved sophisticated mechanisms to complete development in their hosts, including suppression and evasion of immunity at multiple levels (Maizels and McSorley, 2016). *Heligmosomoides polygyrus bakeri* (*H. polygyrus*) is a natural parasite of mice which is used as a laboratory model of intestinal nematode infection (Behnke et al., 2009), and has proven invaluable in defining mechanisms responsible for suppression of anti-parasite immunity and associated amelioration of inflammatory disease (Reynolds et al., 2012). Type 2 immunity leads to an M2 macrophage population which, in concert with antibodies (McCoy et al., 2008) appear to be the principle effector cells which drive expulsion of *H. polygyrus* during secondary infection (Patel et al., 2009). During primary infection these effector mechanisms are largely kept in check by induction of a strong regulatory T cell (Treg) population (Finney et al., 2007) which allows the parasite sufficient time to reproduce and complete its life cycle.

Total secreted products from adult *H. polygyrus* (HES; ***H****eligmosomoides* **E**xcretory-**S**ecretory products) recapitulate the immunomodulatory effects of live infection (Maizels et al., 2012). This observation stimulated subsequent purification of individual proteins and definition of their effector mechanisms, such as an Alarmin Release Inhibitor (HpARI) which tethers IL-33 in necrotic cells, preventing its release and biological activity (Osbourn et al., 2017), and a TGFβ mimic (HP-TGM) which expands the host Treg population (Johnston et al., 2017). These represent a small proportion of the overall secretome however: a proteomic analysis of the products secreted by adult *H. polygyrus* identified 374 individual proteins by LC–MS/MS, many of these members of expanded gene families with as yet undefined functions (Hewitson et al., 2011).

Included in this dataset were proteins identified as apyrases, enzymes which feature prominently in secretomes from numerous parasitic nematode species. Apyrases (ATP

diphosphohydrolases, EC 3.6.1.5) are nucleotide-metabolising enzymes defined as those which catalyse the hydrolysis of nucleoside triphosphates (NTPs) and nucleoside diphosphates (NDPs) to nucleoside monophosphates (NMPs) and inorganic phosphate (Plesner, 1995). In vertebrates, apyrases are primarily membrane-bound enzymes of the CD39 family, often referred to ecto-ATPases, which act to hydrolyse extracellular ATP and ADP, with diverse roles in regulation of neurotransmission, cell growth, pain, haemostasis and immune cell function (Bele and Fabbretti, 2015; Cekic and Linden, 2016; Zimmermann, 2016). A second, structurally distinct class of apyrases is represented by SCANs (**S**oluble **C**alcium-**A**ctivated **N**ucleotidases), now generally referred to as CANTs (**C**alcium-**A**ctivated **N**ucleo**T**idases). These enzymes were first identified in salivary glands of the blood-feeding bed bug *Cimex lectularius* (Valenzuela et al., 2001) but are expressed by numerous invertebrate and vertebrate organisms, including nematodes (Nisbet et al., 2012; Uccelletti et al., 2004; Zarlenga et al., 2011) and humans (Smith et al., 2002).

Although there are numerous facets to purinergic regulation of immunity, many of these are linked to the dichotomy of ATP acting as an alarmin, promoting inflammation by acting on P2X and P2Y purinergic receptors expressed on multiple cells, whereas adenosine, acting through P1 receptors and in particular the A2A receptor, is broadly anti-inflammatory (Cekic and Linden, 2016; Ohta and Sitkovsky, 2014).

Proteomic analysis identified four apyrases (APY-1–APY-4) in the secreted products of adult *H. polygyrus,* with APY-1 consisting of four minor isoforms (Hewitson et al., 2011). Subsequently, L4 stage *H. polygyrus* were shown to secrete APY-3 and a fifth enzyme termed APY-5 (Hewitson et al., 2013). Given the multiple forms of putative apyrases secreted by the parasite, we sought to define their enzymatic properties in order to determine whether they might perform distinct or redundant functions, and assess their potential to stimulate protective immunity via vaccination.

## 2. Material and methods

### *2.1. Ethics statement*

This study was approved by the Animal Welfare Ethical Review Board at Imperial College London, and was licensed by and performed under the UK Home Office Animals (Scientific Procedures) Act Personal Project Licence number 70/8193: 'Immunomodulation by helminth parasites'.

### *2.2. Isolation of parasites and secreted material*

*H. polygyrus* were maintained in female CBA x C57Bl/6 F1 mice by oral gavage with 400 infective larvae. Fourth stage larvae were recovered at day 5 and adult worms at day 14 post-infection. For collection of HES, adults were washed extensively in phosphate-buffered saline (PBS) and cultured in serum-free medium as previously described (Johnston et al., 2015) for 2 weeks, removing medium every 2 days. Pooled medium was concentrated over a 3000 MW cutoff Amicon membrane, washed extensively with PBS, protein concentration determined, aliquoted and stored at −80 °C. Third stage (infective) larvae were activated by exsheathment and subsequent culture for 24 hrs in RPMI 1640 plus 2% mouse serum and antibiotics at 37ºC in 5% $CO_2$. Exsheathment was performed by bubbling 40% $CO_2$ in nitrogen into saline for 5 min, the pH adjusted to pH 2.0 with HCl, adding the acidified saline to larvae, bubbling through again with 40% $CO_2$ in nitrogen for 30 secs, sealing the tube and incubating at 37°C for a further 30 min (Sommerville and Bailey, 1973).

### *2.3. Analysis of apyrase protein sequences*

Amino acid sequences of the five apyrases (GenBank Accession numbers JF721963, JF721965, JF721966, JF721967, WormBase Parasite identifier HPOL_0000506701-mRNA-1) were aligned with human CANT-1 (NM_138793.4) using Clustal Omega (Madeira et al., 2019). Phylogenetic analysis of the *H. polygyrus* apyrases, including the minor sequence variants observed for APY-1, was performed using MEGA X https://www.megasoftware.net (Kumar et al., 2018) and an inferred evolutionary tree drawn using the Neighbour-Joining method (Saitou and Nei, 1987). Residues with known roles in substrate binding and catalysis were identified by comparison with structural and mutagenic analysis of human CANT-1 (Dai et al., 2004).

*2.4. Quantitative Real-time Polymerase Chain Reaction (qPCR)*

Total RNA was extracted from L3 infective larvae, activated L3, L4 and adult worms by homogenising in 1 ml TRIzol reagent (Sigma), using a Tissuelyser II (Qiagen) and converted to cDNA using iScript (Bio-Rad). The qPCR reactions were carried out using PowerUp SYBR Green Master Mix (ThermoFisher) in a 7500 Fast Real-time PCR thermocycler (Applied Biosystems) under the following conditions: 30 seconds denaturation at 95°C, 30 seconds annealing at 60°C, 30 seconds elongation at 72°C for 40 cycles. All reactions were run in duplicate, with no-template and no-Reverse Transcription (RT) controls. Relative expression of each apyrase was calculated by the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) using *iscu-1* and *tub-α* as reference genes. Primer sequences were as follows (sense / antisense): *apy-1* 5′-CGCACCTAAACATGAAAGGACGA-3′/ 5′-GCCCAGAATTGAGGAACAGCC-3′; *apy-2* 5′-ATGCCATGAAAGCCGAGTGG-3′ / 5′-TCCATCGGGCGATACAACCT-3′; *apy-3* 5′-AACGGCATGAAAGCGGAGTG-3′ / 5′-GAACAGCACCATCAGCCGAG-3′; *apy-4* 5′-TCGTGTCGCCTGAAGGAGTT-3′ / 5′-CGTCGTACGCCTCTATGGAG-3′; *apy-5* 5′-AAAATCGTCTCGCCAAGCGG-3′ / 5′-

AATGTCTGACCACTGCACCG-3′; *iscu-1* 5′-TGCAATACAAGCGGCGTTGA-3′ / 5′-TCTCAGTGCACGTATCGGCT-3′ and *tub-α* 5′-GCCCTGGAGAAGGACTATGAAGA-3′ / 5′-CGAGTGAGATCTGCTTGACAGC-3′.

*2.5. Expression and purification of recombinant apyrases*

Total RNA was isolated from L4 and adult *H. polygyrus* as described, reverse transcribed to cDNA and used to amplify full-length coding sequence (minus signal peptide) by RT-PCR for each gene (*apy-1.3, apy-2, apy-3, apy-4,* and *apy-5*), introducing 5′ KpnI and 3′ XbaI restriction sites. Primer sequences were as follows (sense / antisense), with lower case indicating nucleotides added for cloning purposes: *apy-1* 5′-gatcatcggtaccAGCCCTTTGCCAGTGGGA-3′ / 5′-gtcatgtctagagcCAAAAAGTACAATCCTTCGAATTTGAGGT-3′; *apy-2* 5′-gatcatcggtaccGCAGTTATCAAGCCCAGAAAGATC-3′ / 5′-gatagctctagagcAATAAAGTATAACCCTTCGAATTTGTGATT-3′; *apy-3* 5′-gatcatcggtaccGCTGCCCCTATGCCTCAG-3′ / 5′-gtcatgtctagagcCTTTTTGACAATGTACAGTCCCTCG-3′; *apy-4* 5′-gatcatcggtaccGCTCCTCTGACGACAGAG-3′ / 5′-gtcatgtctagagcCAAAAAGTACAGTCCTTCAAACTTG-3′; *apy-5* 5′-atcatcggtaccGCTGCCCCTTTGACTCC-3′ / 5′-gtcatgtctagagcTACAAAGTACAGACCCTCAAACT-3′.

Following cleavage, cDNAs were cloned into pPICZα-A downstream of the coding sequence for the *Saccharomyces cerevisiae* α-mating secretion factor, and *Pichia* pastoris X-33 transformed. Expression was optimised for single colonies, then scaled up following the EasySelect Pichia Expression protocol (Invitrogen). His-tagged proteins were purified from

yeast supernatants by Ni-NTA resin affinity chromatography as previously described (Hussein et al., 1999) and protein concentration determined by Bradford assay (Coomassie Protein Assay Kit, Thermo Fisher Scientific). N-linked glycans were removed by digestion with PNGase F according to the manufacturer's (New England BioLabs) protocol.

### *2.6. Measurement of enzymatic activity*

Enzymatic activity was determined using a phosphate colourimetric assay kit (Abcam) assaying inorganic phosphate ($P_i$) released from nucleotides with reference to a standard curve. Substrates tested were adenosine triphosphate (ATP), diphosphate (ADP) and monophosphate (AMP), guanosine triphosphate (GTP), diphosphate (GDP) and monophosphate (GMP), uridine triphosphate (UTP), diphosphate (UDP) and monophosphate (UMP), and cytidine triphosphate (CTP), diphosphate (CDP) and monophosphate (CMP). Reactions were carried out in triplicate in 25 mM HEPES, 150 mM NaCl, pH 7.5 for 10 min at room temperature, using 1 mM nucleotide substrates unless otherwise indicated, terminated and incubated in the dark for 30 min before measuring absorbance at 600 nm using a FLUOstar OPTIMA microplate reader (BMG Labtech). Enzyme activity was expressed as nmol of $P_i$ generated per µg of protein per hr.

To determine divalent cation dependence, $CaCl_2$, $MgCl_2$ or $ZnCl_2$ were added at concentrations up to 4 mM. For optimum pH determination, reactions were carried out in a buffer containing 25 mM Bis-Tris propane and 150 mM NaCl with pH ranging from 5.0 to 10.5 in increments of 0.5 pH units. To determine kinetic constants, assays were carried out in HEPES buffer containing different ATP concentrations up to 4 mM, and kinetic parameters $K_m$ (Michaelis-Menten constant), $K_{cat}$ (turnover rate), and $K_{cat}/K_m$ (catalytic efficiency) were calculated for the five enzymes, by non-linear regression analysis fitted to the Michaelis–Menten equation using Graphpad Prism 8.0 software.

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196

197 *2.7. SDS-PAGE and western blotting*

198 Protein samples were resolved by SDS-12% polyacrylamide gel electrophoresis under
199 standard conditions and gels stained with Coomassie brilliant blue. For western blotting,
200 proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, incubated for 1 hr
201 at room temp in PBS, 5% skimmed milk, 0.05% Tween 20 (blocking buffer) and probed with
202 murine anti-c-myc primary antibody (9E10, Thermo Fisher Scientific) diluted 1:1000 in
203 blocking buffer. The membrane was washed, incubated with goat anti-mouse Ig-horseradish
204 peroxidase secondary antibody (Thermo Fisher Scientific), processed and visualized using
205 enhanced chemiluminescence western blotting detection reagents (Amersham Bioscience).

206

207 *2.8 Immunisation experiments*

208 Female BALB/c mice (6-8 weeks old, Charles River UK) were immunised
209 intraperitoneally with PBS, a cocktail of all 5 apyrases (12.5 µg of each enzyme), or 12.5 µg
210 total HES in Imject Alum adjuvant (Thermo Fisher, 100 µl Alum + 100 µl antigen solution),
211 and boosted with PBS, APY cocktail (2.5 µg of each enzyme) or 2.5 µg HES in alum on days
212 28 and 35 post-immunisation. At day 42, mice were challenged with 250 *H. polygyrus* infective
213 larvae in 200 µl of distilled water by oral gavage. Egg production was determined at days 14,
214 21 and 28 post-challenge, and adult worms recovered and counted at day 28. Antibody
215 responses to immunisation were carried out in separate experiments by bleeding mice 7 days
216 after the second boost, i.e. day 42. IgG from the sera of mice immunised with the apyrase
217 cocktail or PBS controls was extracted via Magne Protein G beads (Promega). It was then used
218 to test possible inhibition of enzymatic activity by addition of 1 µg in a reaction volume of 40

219 µl (i.e. final concentration of 25 µg $ml^{-1}$ IgG) under standard assay conditions (1 mM ATP, 0.5
220 mM $Ca^{2+}$, pH 7.5).
221
222 *2.9. Antibody subtype ELISA*
223 Antigen-specific antibodies were measured by ELISA, coating Nunc 96 well plates
224 with 5 µg $ml^{-1}$ of either individual apyrases or apyrase cocktail in 0.1 M carbonate buffer pH
225 9.6. Titration curves were constructed using serially-diluted sera from immunised or infected
226 mice, and the end point titre determined as the reciprocal dilution at which the optical density
227 dropped below background. HRP-conjugated goat anti-mouse immunoglobulin subclass
228 antibodies (Invitrogen) were diluted in PBS, 1% bovine serum albumin (BSA) at the following
229 dilutions: IgG1 1:12,000, IgG2a 1:8000, IgG2b 1:2000, IgG3 1:8000.
230
231 *2.10. Statistical analysis*
232 Data were expressed as the mean ± SEM and analysed using GraphPad Prism 7.0
233 (GraphPad Software). Significance differences were calculated using one-way ANOVA with
234 Tukey's multiple comparison test: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.
235
236
237 **3. Results**
238
239 *3.1 Primary structure and relative expression of H. polygyrus secreted apyrases*
240
241 The *H. polygyrus* family of apyrases were all closely related to soluble calcium-
242 activated nucleotidases (CANTs), and clearly distinct from CD39 family ecto-nucleotidases.
243 All *H. polygyrus* apyrases had predicted signal peptides as defined by SignalP 5.0 (Almagro

Armenteros et al., 2019) consistent with their identification in parasite secreted products, and either one or two potential N-linked glycosylation sites. Alignment of the five parasite sequences with human CANT-1 is shown in Fig. 1A, with one representative isoform for APY-1 (APY-1.3). The structure of human CANT-1 bound to a substrate analogue has been determined, which delineated the nucleotide- and calcium-binding sites (Dai et al., 2004). The $Ca^{2+}$ ion lies in the middle of a central tunnel in the enzyme, coordinated by the carbonyl oxygen atoms of five amino acids (Ser100, Glu147, Glu216, Ser277 and Glu328) and making contact with the carboxylate group of Asp101, linking five blades of a β-propeller structure and stabilising the enzyme structure (Dai et al., 2004). Fig 1A shows that all six of these residues (boxed) are strictly conserved in the five *H. polygyrus* apyrases, with the exception of Ser277, which is substituted by alanine in APY-5. The residues important for nucleotide binding in CANT-1 are largely conserved (Fig. 1, shaded grey). Mutation of three negatively charged residues in the nucleotide-binding site (Asp44, Asp114 and Glu147) to Asn, Asn and Gln respectively reduced the catalytic rate constant by about 1000-fold, reinforcing the authors' proposition that these amino acids played a role in nucleophilic attack on the β-phosphate group of the nucleotide substrate (Dai et al., 2004), and it is notable that these residues are strictly conserved in all five parasite enzymes (Fig. 1, indicated with a + sign).

Fig. 1B shows that APY-4 is most closely related to APY-1 isoforms (approximately 70% identity), APY-3 most closely related to APY-5 (66% identity), with APY-2 more of an outlier (51-55% identity to the other enzymes). Expression levels of each apyrase in different life cycle stages of the parasite was determined by qPCR. Fig. 1C shows that each enzyme was only expressed by the L4 and adult stages, maximally expressed in the latter, and was undetectable in L3 and activated L3 (only the former shown for clarity). Figs. 1D and 1E show relative gene expression of each apyrase in L4 and adults respectively: in each case *Apy-3* was the most highly expressed enzyme, over 3x greater than that of any other enzyme in adults,

269 although *Apy-5* was also highly expressed in L4. Although not previously detected by
270 proteomic analysis, *Apy-5* transcripts were present in adult worms (Fig. 1E).
271
272 *3.2 Biochemical characterisation of recombinant apyrases*

273 *H. polygyrus* apyrase genes were cloned into the plasmid pPICZαA and expressed as
274 secreted recombinant proteins in *Pichia pastoris* X-33. The proteins were purified from yeast
275 culture supernatants by His-tag affinity chromatography. They had a molecular mass between
276 45 and 60 kDa based on migration in SDS polyacrylamide gels, which was reduced to 38-40
277 kDa by treatment with N-glycanase indicating that, as expected, they were glycosylated in
278 *Pichia* (Fig. 2A). The pPICZαA vector encodes a C-terminal c-myc epitope just before the
279 polyhistidine tag, which allows confirmation of complete read-through of the coding sequence.
280 Correspondingly, all proteins reacted with the anti-c-myc antibody in immunoblot analysis
281 (Fig. 2B).
282 The effect of divalent cations on the catalytic activity of the purified apyrases was tested
283 using ATP as substrate in the presence of calcium, magnesium or zinc as co-factors. Results
284 showed that the addition of $Ca^{2+}$ dramatically enhanced the hydrolysis of ATP, saturating at
285 concentrations between 0.05 and 0.5 mM $CaCl_2$ (Fig. 3A), and no activity was detected in the
286 presence of equivalent $Mg^{2+}$ and $Zn^{2+}$ concentrations (data not shown). All secreted apyrases
287 were therefore strictly calcium-dependent.
288 The influence of pH on ATP hydrolysis was then investigated. All enzymes showed
289 optimal activity in alkaline conditions over a broad range between pH 7.0 and 10.5 (Fig. 3B).
290 All subsequent experiments were performed under standard conditions of pH 7.5 in the
291 presence of 0.5 mM $CaCl_2$. Enzyme activities was further characterised, examining
292 specificities with a variety of substrates at a concentration of 1 mM. As shown in Fig. 4, all
293 apyrases exhibited a broad substrate specificity, hydrolysing only nucleoside di- and

294 triphosphates, but with no radical preference for any individual nucleoside and no major
295 difference between the enzymes. The *H. polygyrus* enzymes thus belong to the calcium-
296 dependent nucleotidase family, and are active over a broad pH range. Kinetic parameters for
297 hydrolysis of ATP were determined for the five enzymes, which showed that Apy-3 and Apy-
298 2 had the highest catalytic efficiency (Table 1).

299 Total secreted products (HES) were collected from adult *H. polygyrus* and the
300 nucleotidase activity determined. Native apyrases showed essentially the same properties as
301 the recombinant enzymes in terms of nucleotide specificity, cation dependence and pH
302 optimum, in addition to demonstrating that *H. polygyrus* does not secrete a 5′-nucleotidase
303 capable of hydrolysing nucleoside monophosphates (Fig. 3, 4). For clarity, AMP is the only
304 nucleoside monophosphate shown in Fig 4, but no activity was detected for any enzyme or
305 HES against AMP, UMP, GMP and CMP.

306

307 *3.3 Immunisation with apyrase cocktail induces partial protection against challenge infection*

308

309 To test whether the apyrases were capable of inducing protective immunity in a vaccine
310 formulation, mice were immunised with a cocktail of all five enzymes, challenged by infection
311 with *H. polygyrus* seven days after the second boost, and faecal egg output and worm burdens
312 determined. Animals immunised with the apyrase cocktail showed reduced egg output at day
313 28 post-infection, and a lower burden of adult worms at the same time point (Fig. 5).
314 Reductions in egg output and adult worms numbers were only partial however, in contrast to
315 immunisation with HES, which triggered sterile immunity against challenge (Fig. 5), as had
316 previously been demonstrated (Hewitson et al., 2013).

317

318 *3.4 Antibody response to apyrases in naturally infected and immunised mice*

Polyclonal IgG antibodies have been proposed to limit egg production by adult *H. polygyrus* during primary infection, with affinity-matured parasite-specific IgG and IgA preventing parasite development following multiple infections (McCoy et al., 2008). The sterile immunity elicited by vaccination with *H. polygyrus* secreted products is dependent on the concerted action of parasite-specific IgG1 and myeloid cells, and antibody-mediated neutralisation of immunomodulatory molecules is thought to be an important factor in promoting this (Hewitson et al., 2015). We thus examined the antibody response to apyrases following natural infection or immunisation. Natural infection provoked very low titres of apyrase-specific antibodies, with no detectable IgG2a, and the highest level of antibodies directed to APY-3 (Fig. 6A). As expected, immunisation with the apyrase cocktail evoked much higher titres of antibody, principally IgG1 and IgG2b (Fig. 6B), although IgG purified from these mice showed no inhibition of enzyme activity when used at 25 μg $ml^{-1}$ in a standard assay for ATP hydrolysis (data not shown). Immunisation with HES did not elicit significant levels of antibodies to apyrases (data not shown).

## 4. Discussion

Most known apyrases are related in terms of primary structure to CD39, an integral plasma membrane protein with extrinsically-oriented nucleoside triphosphate diphosphohydrolase activity (Maliszewski et al., 1994; Plesner, 1995; Wang and Guidotti, 1998). A second, evolutionarily distinct and widespread class of apyrases is represented by secreted, calcium-dependent enzymes initially identified in *Cimex* salivary glands (Devader et al., 2006; Ribeiro and Valenzuela, 2003; Smith et al., 2002; Valenzuela et al., 1998). The *H. polygyrus* enzymes, like all nematode apyrases identified thus far, belong to this latter group.

Given their potential role in modulation of host immunity, we sought to determine their biochemical characteristics, particularly in terms of substrate specificity.

Their activity indicates that they can be classified as $Ca^{2+}$-dependent apyrases with a broad, largely alkaline pH optimum, and strict conservation of the residues known to coordinate calcium binding in human CANT-1 is consistent with this. They differ from the *O. ostertagia* secreted apyrase, which has a pH optimum between 6.0 and 7.0, although expression of this enzyme is restricted to fourth stage larvae, which inhabit the gastric gland of their bovine host (Zarlenga et al., 2011). It is possible that the broader pH optimum of the *H. polygyrus* enzymes reflect existence in both the intestinal lumen and mucosal tissue, although interesting to note that the tegumental apyrase of *Schistosoma mansoni*, a membrane-bound ecto-enzyme related to CD39 with externally-oriented activity, also has a broad alkaline pH optimum very similar to the *H. polygyrus* enzymes (Da'dara et al., 2014).

*H. polygyrus* apyrases possess a broad substrate specificity catalysing the hydrolysis of all nucleoside tri- and diphosphates, with an overall slight preference for adenine substrates, but no activity on nucleoside monophosphates. Blood-feeding arthropod salivary apyrases strongly favour ADP and ATP. This is to be expected, as ADP is a potent physiological agonist for platelet aggregation (Daniel et al., 1998), and the primary function of these apyrases is assumed to be inhibition of haemostasis and facilitation of blood-feeding. *Schistosoma mansoni* has a suite of tegumental ecto-enzymes which degrade exogenous nucleotides, two of which (an apyrase and an ectonucleotide pyrophosphatase) hydrolyse ADP, whereas the apyrase also hydrolyses ATP (Da'dara et al., 2014; Elzoheiry et al., 2018). Given the intravascular location of the adult parasite, inhibition of haemostasis is extremely important for *S. mansoni*. The ectonucleotide pyrophosphatase has been shown to inhibit platelet aggregation (Elzoheiry et al., 2018), and RNAi knockdown experiments have established that this enzyme is an important contributor to establishment of infection (Bhardwaj et al., 2011). Vertebrate

CANTs, in contrast, may have little or no activity against ADP, and have a preference for UDP (Smith and Kirley, 2006). This, and their location in the endoplasmic reticulum and Golgi complex, is indicative of a role in glycosylation via nucleotide sugar/nucleotide monophosphate exchange, and CANT-1 knockout mice have severe defects in proteoglycan biosynthesis (Paganini et al., 2019).

The pro-inflammatory role of nucleoside di- and triphosophates and the anti-inflammatory activity of adenosine (Cekic and Linden, 2016) has long been thought to be the rationale for secretion of nucleotide-metabolising enzymes by parasitic nematodes (Gounaris, 2002; Gounaris and Selkirk, 2005). However, the lack of activity of the *H. polygyrus* apyrases on nucleoside monophosphates means that, operating independently, the parasite enzymes cannot give rise to adenosine. Generation of adenosine is utilised as a mechanism of immunosuppression by Tregs, requiring CD39 to first generate adenosine monophosphate, and CD73, a 5′-nucleotidase, to convert this into adenosine (Borsellino et al., 2007; Deaglio et al., 2007). This requires coordinated high levels of expression of both enzymes and close cell-cell contact in order to generate requisite levels of pericellular adenosine. A2A receptor engagement induces T cell anergy, however adenosine is not only involved in the effector mechanism of suppression, but also actively promotes the development and expansion of Treg populations (Ohta et al., 2012; Zarek et al., 2008).

Whilst this might appear an attractive rationale for secretion of apyrases by nematode parasites, no 5′-nucleotidase activity is present in secreted products of adult parasites (Fig. 4). Production of adenosine by nematode parasites might also be disadvantageous in that in the context of a type 2 cytokine environment, adenosine has been shown to augment expression of markers identified with an M2 phenotype in macrophages (Csoka et al., 2012). Moreover, mice deficient in the A2A receptor showed impaired expulsion of secondary *H. polygyrus* infection, accompanied by lower production of IL-4 and IL-13, and reduced recruitment of M2

394 macrophages and eosinophils, suggesting that adenosine acts as an alarmin for type 2 effector
395 mechanisms necessary for expulsion of parasites from the intestinal tract (Patel et al., 2014).
396 As the end products of nucleotide catabolism by *H. polygyrus* apyrases are nucleoside
397 monophosphates, the most likely role of these enzymes is simply removal of nucleoside
398 triphosphates, and ATP in particular.

399 Nucleoside triphosphates and diphosphates have a number of signalling activities
400 which could be deleterious to nematode parasites, for instance UDP can activate the P2Y6
401 receptor on monocytes leading to the production of IL-8 (von Kugelgen and Wetter, 2000), and
402 P2Y6 on intestinal epithelial cells, leading to $Cl^-$ flux and fluid secretion (Kottgen et al., 2003).
403 However, the most pivotal role for extracellular nucleotides in immunity is likely to be the role
404 of ATP in promoting release of IL-33. In vitro and in vivo experiments with extracts of the
405 fungus *Alternaria alternata* showed that the primary effect of exposure to *Alternaria* was ATP
406 release within 60 seconds, which subsequently promoted release of IL-33 1 hr later (Kouzaki
407 et al., 2011). This was confirmed by pharmacological inhibition of P2 receptors and the use of
408 knockout mice, which indicated that ATP exerted this effect principally through the P2Y2
409 receptor (Kouzaki et al., 2011).

410 IL-33 is a critical initiator of allergic responses and type 2 immunity (Liew et al., 2016),
411 and its role in immunity to nematodes has been highlighted by a study using Spi-B-deficient
412 mice, which have elevated numbers of mast cells and accelerated expulsion of *H. polygyrus*
413 (Shimokawa et al., 2017). Mast cells were shown to produce IL-33 in response to ATP released
414 from apoptotic intestinal epithelial cells, and blockade of the P2X7 ATP receptor suppressed
415 ATP release, activation of type 2 innate lymphoid cells (ILC2s) and worm expulsion
416 (Shimokawa et al., 2017). Co-administration of HES with *Alternaria* suppressed induction of
417 type 2 immunity in the airways (McSorley et al., 2014). The authors subsequently identified
418 the mediator HpARI, which binds to and inhibits cellular release of IL-33, as a key factor in

this inhibition (Osbourn et al., 2017). Helminth parasites commonly use overlapping, seemingly redundant mechanisms to suppress immunity, and it is thus likely that apyrases also contribute to suppression of IL-33 by hydrolysis of extracellular ATP. We tested this by several different approaches, including intranasal administration of recombinant enzymes with *Alternaria* extracts. We could not demonstrate any inhibition of induction of type 2 immunity mediated by parasite apyrases, although this may be restricted by delivery of sufficient enzyme to the appropriate site in vivo (data not shown).

Immunisation of mice with the full complement of adult *H. polygyrus* secreted proteins (HES) generates sterile immunity (Hewitson et al., 2013), and a cocktail of three selected native proteins, VAL-1, -2 and -3 generates almost complete protection (Hewitson et al., 2015). In the latter study larvae were trapped by myeloid cells in the submucosal tissues of the small intestine, preventing their maturation. This was dependent on specific IgG1 antibodies independent of FcR signalling, suggesting that neutralisation of putative immunomodulatory activities may underlie their effectiveness as immunogens, although this is difficult to assess without knowledge of their function (Hewitson et al., 2015). It was notable that in the *H. polygyrus*/mouse model, HES depleted of VAL-1, -2 and -3 still generated sterile immunity, implying the presence of alternative protective immunogens in secreted products (Hewitson et al., 2015). It was in this context that we assayed the effect of immunisation with the apyrase cocktail. Natural infection provoked very low titres of antibody to specific apyrases. Immunisation with alum elicited very high titres to the apyrase cocktail, yet generated only partial protection against reinfection in terms of lower recovery of adult worms at day 28 p.i. Nevertheless, this is the first report of protection against *H. polygyrus* infection by immunisation with recombinant proteins, and it is perhaps notable that IgG from immunised mice did not inhibit enzyme activity. Whilst it is possible that addition of large N-linked glycans to recombinant proteins expressed in *Pichia* might block antibodies which could

444 otherwise neutralise enzymatic activity, use of a system which facilitates correct protein
445 folding is important in generating antibodies which optimally bind native proteins, and it is
446 notable that Apy-1 and -4 are not hyperglycosylated (Fig. 2).

447 It is unclear why *H. polygyrus* secretes five enzymes with such similar properties,
448 although secretion of multiple isoforms of acetylcholinesterase with the same substrate
449 specificities and general characteristics by *Nippostrongylus brasiliensis* has also been observed
450 (Hussein et al., 2002). The rationale for such families may thus not lie in distinct properties
451 and function, but in simple diversification. This could favour avoidance of neutralising
452 antibodies to a given activity, although no evidence for this was noted in the current study.
453 Thus, even if the apyrases act to dampen induction of type 2 immunity by hydrolysis of ATP
454 from damaged cells during natural infection, neutralisation of enzymatic activity does not
455 appear to contribute to the immunity afforded by secreted protein vaccine preparations.

456

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462

463

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635
636

637 **Figure Legends**

638

639 **Fig. 1.** The *H. polygyrus* secreted apyrase family. (A) Alignment of *H. polygyrus* apyrases
640 (APY-1 - APY-5; APY-1.3 shown as a representative isoform of APY-1) with human CANT-
641 1 (HsCANT-1). GenBank Accession numbers/WormBase Parasite identifier APY-1.3:
642 JF721963; APY-2: JF721965; APY-3: JF721966; APY-4: JF721967; APY-5:
643 HPOL_0000506701-mRNA-1; HsCANT-1: NM_138793.4. Alignment was performed with
644 Clustal Omega. Predicted signal peptides are underlined at the N-terminus of each protein, and
645 potential N-linked glycosylation sites in the parasite enzymes are also underlined. Residues
646 important for nucleotide and $Ca^{2+}$ binding in CANT-1 and predicted counterparts in *H.*
647 *polygyrus* apyrases are boxed and shaded grey respectively. The three residues predicted to
648 play a role in nucleophilic attack on the β-phosphate group of the nucleotide substrate are
649 indicated with a plus (+) sign. (B) Phylogenetic tree of *H. polygyrus* apyrases. Analysis
650 performed using MEGA X and Neighbour-Joining. The tree is drawn to scale with branch
651 lengths corresponding to inferred evolutionary distances. (C-E) Expression levels of apyrases
652 determined by qPCR analysis. (C) Relative gene expression in different life cycle stages for
653 each apyrase, with that in adults set at 100%. (D) Relative gene expression of each apyrase in
654 L4, with that of *Apy-3* set at 100%. (E) Relative gene expression of each apyrase in adults, with
655 that of *Apy-3* set at 100%. Results are expressed as means +/- SEM of duplicate measurements
656 from three independent experiments.

657

658 **Fig. 2.** Expression of apyrases in *Pichia pastoris*. Proteins were purified from yeast culture
659 supernatants by His-tag affinity chromatography, digested (+) or not (-) with PNGase F,
660 resolved by SDS-PAGE and (A) stained with Coomassie Blue or (B) blotted and probed with
661 mouse anti-c-myc antibody. Migration of molecular weight markers is shown in kDa.

662

663 **Fig. 3.** Calcium dependence and pH optima of recombinant apyrases. (A) Calcium dependence
664 was determined using ATP (1 mM) as substrate with different concentrations of $CaCl_2$ (0-2
665 mM). (B) The pH optimum of was determined using a pH range from 5.0-10.5, in the presence
666 of 0.5 mM $Ca^{2+}$ and 1 mM ATP. Reactions were carried out in triplicate. Data show the mean
667 +/- SEM at each point and are representative of 3 independent experiments. Activity of total
668 native adult secreted products (HES) is shown for comparison.

669

670 **Fig. 4.** Substrate specificity. The substrate specificity of recombinant enzymes was determined
671 using nucleoside triphosphates, diphosphates or monophosphates at a concentration of 1 mM
672 in the presence of 0.5 mM $Ca^{2+}$ as described in Materials and methods. Reactions were carried
673 out in triplicate. Data show the mean + SEM and are representative of 3 independent
674 experiments. Activity of total native adult secreted products (HES) is shown for comparison.
675 For clarity, AMP is the only nucleoside monophosphate shown in Fig 4, but no activity was
676 detected for any enzyme or HES against AMP, UMP, GMP and CMP.

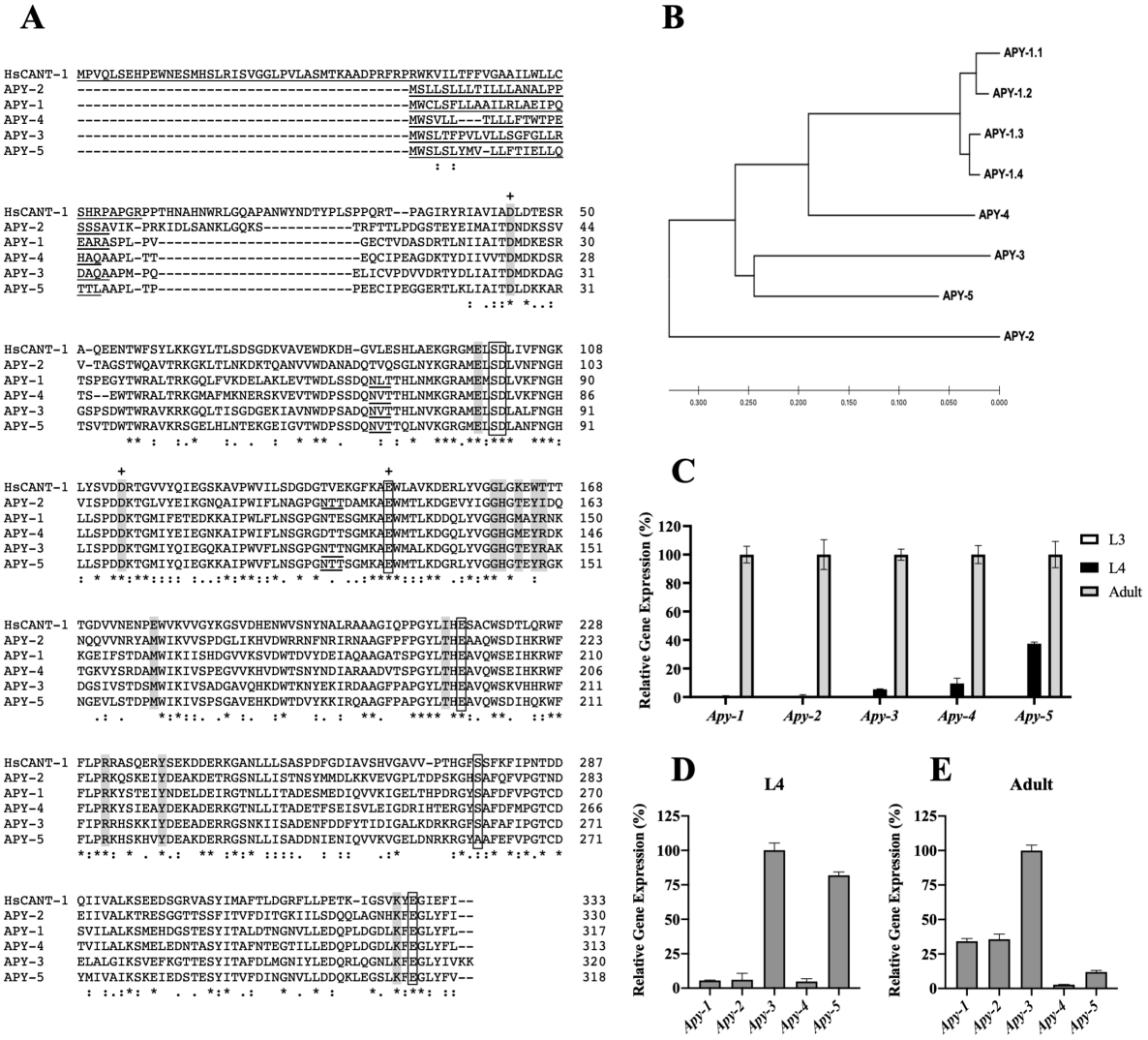
677

678 **Fig. 5.** Vaccination with a cocktail of apyrases induces partial protection against challenge
679 infection. (A) Eggs per gram (epg) faeces at d 14, 21 and 28 post-challenge. (B) Adult worm
680 recoveries from the small intestine at d 28 post-challenge. Mice were also immunised with PBS
681 or HES under the same regimen. Data are represented as mean +/- SEM (n=8). Data were
682 pooled from two experiments, with statistical significance calculated using the one-way
683 ANOVA test with comparisons to the PBS/Alum group (**$p < .01$; ****$p < .0001$).

684

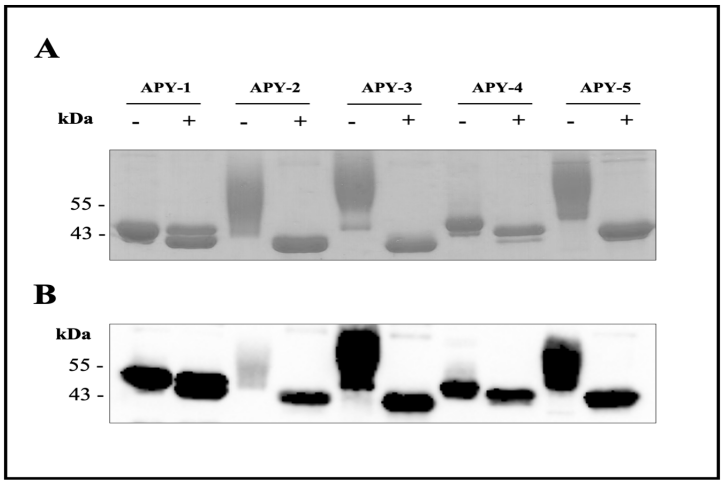
685 **Fig. 6.** Apyrase-specific antibodies induced by natural infection or vaccination. (A) Antigen-
686 specific IgG isotype responses in mice infected with *H. polygyrus*. These were determined at

687 35 days post infection, coating plates with individual apyrases. Data represent the mean end
688 point titre +/- SEM from 6 individual mice. (B) Antigen-specific IgG isotype responses in mice
689 vaccinated with the apyrase cocktail or PBS in alum as outlined in Materials and methods.
690 Mice were bled 7 days after the second boost at day 42, and end point titres to the complete
691 cocktail determined. Data represent the mean +/- SEM from 5 individual mice.
692


A
HsCANT-1 MPVQLSEHPEWNESMHSLRISVGGLPVLASMTKAADPRFRPRWKVILTFFVGAAILWLLC
APY-2 ----------MSLLSLLLTILLLANALPP
APY-1 ----------MWCLSFLLAAILRLAEIPQ
APY-4 ----------MWSVLL---TLLLFTWTPE
APY-3 ----------MWSLTFPVLVLLSGFGLLR
APY-5 ----------MWSLSLYMV-LLFTIELLQ
HsCANT-1 SHRPAPGRPPTHNAHNWRLGQAPANWYNDTYPLSPPQRT--PAGIRYRIAVIADLDTESR 50
APY-2 SSSAVIK-PRKIDLSANKLGQKS----------TRFTTLPDGSTEYEIMAITDNDKSSV 44
APY-1 EARASPL-PV----------GECTVDASDRTLNIIAITDMDKESR 30
APY-4 HAQAAPL-TT----------EQCIPEAGDKTYDIIVVTDMDKDSR 28
APY-3 DAQAAPM-PQ----------ELICVPDVVDRTYDLIAITDMDKDAG 31
APY-5 TTLAAPL-TP----------PEECIPEGGERTLKLIAITDLDKKAR 31
HsCANT-1 A-QEENTWFSYLKKGYLTLSDSGDKVAVEWDKDH-GVLESHLAEKGRGMEISDLIVFNGK 108
APY-2 V-TAGSTWQAVTRKGKLTLNKDKTQANVVWDANADQTVQSGLNYKGRAMEISDLVNFNGH 103
APY-1 TSPEGYTWRALTRKGQLFVKDELAKLEVTWDLSSDQNLTTHLNMKGRAMEMSDLVKFNGH 90
APY-4 TS--EWTWRALTRKGMAFMKNERSKVEVTWDPSSDQNVTTHLNMKGRAMEISDLVKFNGH 86
APY-3 GSPSDWTWRAVKRKGQLTISGDGEKIAVNWDPSADQNVTTHLNVKGRAMEISDLALFNGH 91
APY-5 TSVTDWTWRAVKRSGELHLNTEKGEIGVTWDPSSDQNVTTQLNVKGRGMEISDLANFNGH 91
HsCANT-1 LYSVDDRTGVVYQIEGSKAVPWVILSDGDGTVEKGFKAEWLAVKDERLYVGGLGKEWTTT 168
APY-2 VISPDDKTGLVYEIKGNQAIPWIFLNAGPGNTTDAMKAEWMTLKDGEVYIGGHGTEYIDQ 163
APY-1 LLSPDDKTGMIFETEDKKAIPWLFLNSGPGNTESGMKAEWMTLKDDQLYVGGHGMAYRNK 150
APY-4 LLSPDDKTGMIYEIEGNKAIPWIFLNSGRGDTTSGMKAEWMTLKDGQLYVGGHGMEYRDK 146
APY-3 LISPDDKTGMIYQIEGQKAIPWVFLNSGPGNTTNGMKAEWMALKDGQLYVGGHGTEYRAK 151
APY-5 LLSPDDKTGMIYQIEGKKAIPWVFLNSGPGNTTSGMKAEWMTLKDGRLYVGGHGTEYRGK 151
HsCANT-1 TGDVVNENPEWVKVVGYKGSVDHENWVSNYNALRAAAGIQPPGYLIHESACWSDTLQRWF 228
APY-2 NQQVVNRYAMWIKVVSPDGLIKHVDWRRNFNRIRNAAGFPFPGYLTHEAAQWSDIHKRWF 223
APY-1 KGEIFSTDAMWIKIISHDGVVKSVDWTDVYDEIAQAAGATSPGYLTHEAVQWSEIHKRWF 210
APY-4 TGKVYSRDAMWIKIVSPEGVVKHVDWTSNYNDIARAADVTSPGYLTHEAVQWSEIHKRWF 206
APY-3 DGSIVSTDSMWIKIVSADGAVQHKDWTKNYEKIRDAAGFPAPGYLTHEAVQWSKVHHRWF 211
APY-5 NGEVLSTDPMWIKIVSPSGAVEHKDWTDVYKKIRQAAGFPAPGYLTHEAVQWSDIHQKWF 211
HsCANT-1 FLPRRASQERYSEKDDERKGANLLLSASPDFGDIAVSHVGAVV-PTHGFSSFKFIPNTDD 287
APY-2 FLPRKQSKEIYDEAKDETRGSNLLISTNSYMMDLKKVEVGPLTDPSKGHSAFQFVPGTND 283
APY-1 FLPRKYSTEIYNDELDEIRGTNLLITADESMEDIQVVKIGELTHPDRGYSAFDFVPGTCD 270
APY-4 FLPRKYSIEAYDEKADERKGTNLLITADETFSEISVLEIGDRIHTERGYSAFDFMPGTCD 266
APY-3 FIPRRHSKKIYDEEADERRGSNKIISADENFDDFYTIDIGALKDRKRGFSAFAFIPGTCD 271
APY-5 FLPRKHSKHVYDEAKDERRGSNLLISADDNIENIQVVKVGELDNRKRGYAAFEFVPGTCD 271
HsCANT-1 QIIVALKSEEDSGRVASYIMAFTLDGRFLLPETK-IGSVKYEGIEFI-- 333
APY-2 EIIVALKTRESGGTTSSFITVFDITGKIILSDQQLAGNHKFEGLYFI-- 330
APY-1 SVILALKSMEHDGSTESYITALDTNGNVLLEDQPLDGDLKFEGLYFL-- 317
APY-4 TVILALKSMELEDNTASYITAFNTEGTILLEDQPLDGDLKFEGLYFL-- 313
APY-3 ELALGIKSVEFKGTTESYITAFDLMGNIYLEDQRLQGNLKFEGLYIVKK 320
APY-5 YMIVAIKSKEIEDSTESYITVFDINGNVLLDDQKLEGSLKFEGLYFV-- 318
B
APY-1.1
APY-1.2
APY-1.3
APY-1.4
APY-4
APY-3
APY-5
APY-2
0.300
0.250
0.200
0.150
0.100
0.050
0.000
C
Relative Gene Expression (%)
120
100
80
60
40
20
0
L3
L4
Adult
Apy-1
Apy-2
Apy-3
Apy-4
Apy-5
D
L4
Relative Gene Expression (%)
125
100
75
50
25
0
Apy-1
Apy-2
Apy-3
Apy-4
Apy-5
E
Adult
Relative Gene Expression (%)
125
100
75
50
25
0
Apy-1
Apy-2
Apy-3
Apy-4
Apy-5

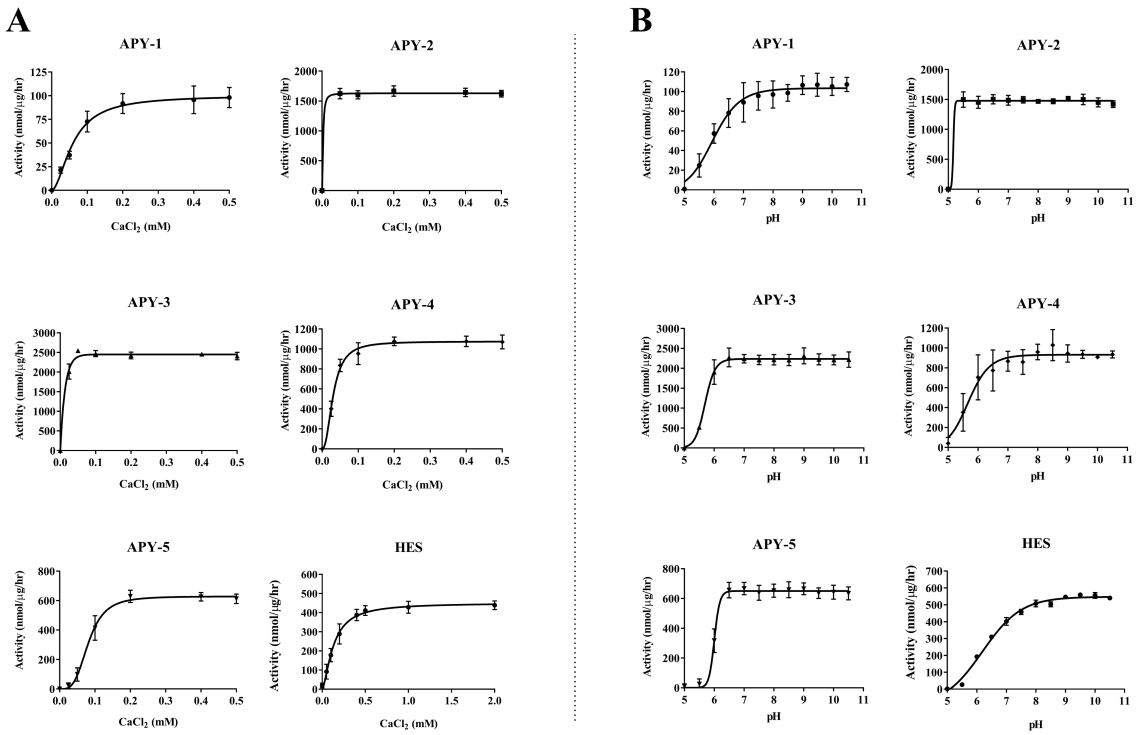
693

694 Fig 1



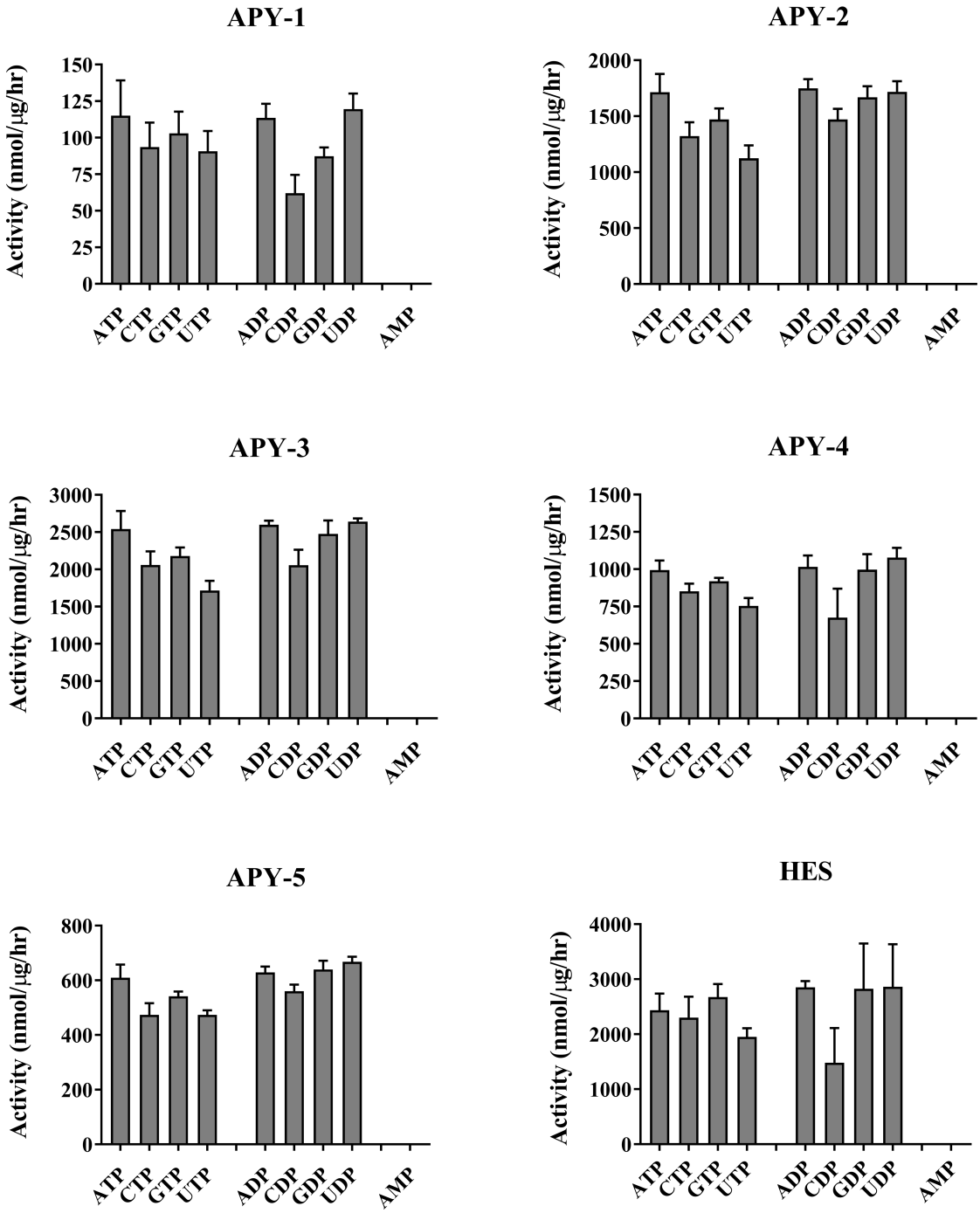

695

696 Fig 2


A
APY-1
APY-2
APY-3
APY-4
APY-5
HES
Activity (nmol/μg/hr)
CaCl2 (mM)
B
APY-1
APY-2
APY-3
APY-4
APY-5
HES
Activity (nmol/μg/hr)
pH

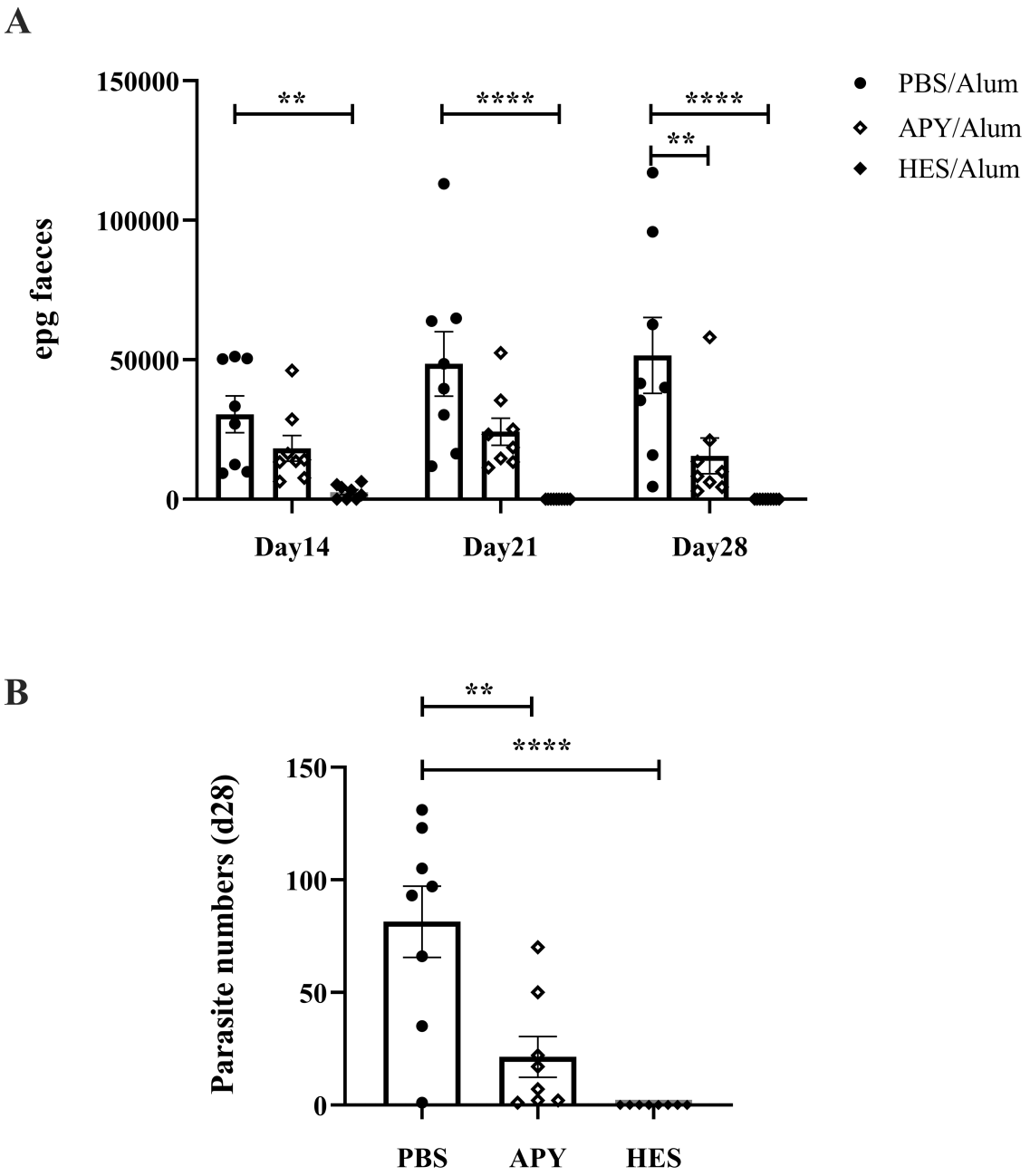
697

698 Fig 3


APY-1
Activity (nmol/μg/hr)
150
125
100
75
50
25
0
ATP
CTP
GTP
UTP
ADP
CDP
GDP
UDP
AMP
APY-2
Activity (nmol/μg/hr)
2000
1500
1000
500
0
ATP
CTP
GTP
UTP
ADP
CDP
GDP
UDP
AMP
APY-3
Activity (nmol/μg/hr)
3000
2500
2000
1500
1000
500
0
ATP
CTP
GTP
UTP
ADP
CDP
GDP
UDP
AMP
APY-4
Activity (nmol/μg/hr)
1500
1250
1000
750
500
250
0
ATP
CTP
GTP
UTP
ADP
CDP
GDP
UDP
AMP
APY-5
Activity (nmol/μg/hr)
800
600
400
200
0
ATP
CTP
GTP
UTP
ADP
CDP
GDP
UDP
AMP
HES
Activity (nmol/μg/hr)
4000
3000
2000
1000
0
ATP
CTP
GTP
UTP
ADP
CDP
GDP
UDP
AMP

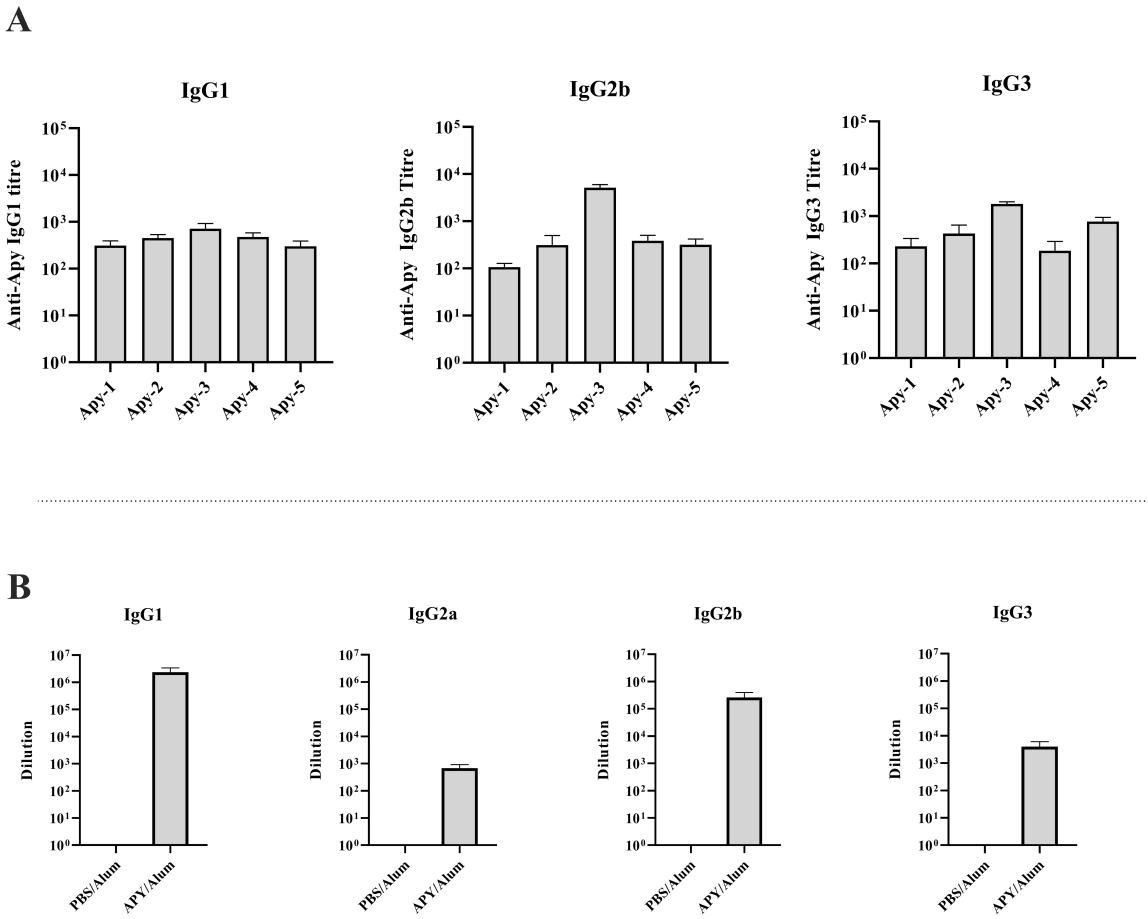
699

700 Fig 4


A
150000
100000
50000
0
epg faeces
**
****
****
**
PBS/Alum
APY/Alum
HES/Alum
Day14
Day21
Day28
B
**
****
150
100
50
0
Parasite numbers (d28)
PBS
APY
HES

701

702 Fig 5


A
IgG1
IgG2b
IgG3
Anti-Apy IgG1 titre
Anti-Apy IgG2b Titre
Anti-Apy IgG3 Titre
Apy-1
Apy-2
Apy-3
Apy-4
Apy-5
B
IgG1
IgG2a
IgG2b
IgG3
Dilution
PBS/Alum
APY/Alum

703

704 Fig 6