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1 **RNAi gene knockdown in the poultry red mite, *Dermanyssus gallinae* (De Geer**
2 **1778), a tool for functional genomics**
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26 ## Abstract

27 **Background:** The avian haematophagous ectoparasite, *Dermanyssus gallinae* or the
28 poultry red mite, causes significant economic losses to the egg laying industry
29 worldwide and also represents a significant welfare threat. Current acaricide-based
30 controls are unsustainable due to the mite's ability to rapidly develop resistance, thus
31 developing a novel sustainable means of control for *D. gallinae* is a priority. RNA
32 interference (RNAi) mediated gene silencing is a valuable tool for studying gene
33 function in non-model organisms, but is also emerging as a novel tool for parasite
34 control.
35
36 **Methods:** Here we use an *in silico* approach to identify core RNAi pathway genes in
37 the recently sequenced *D. gallinae* genome. In addition we utilise an *in vitro* feeding
38 device to deliver dsRNA to *D. gallinae* targeting the *D. gallinae vATPase subunit A*
39 (*Dg vATPase A*) gene and monitor gene knockdown using quantitive PCR (qPCR).
40
41 **Results:** We identified core components of the small interfering RNA (siRNA) and
42 micro RNA (miRNA) pathways in *D. gallinae*, which indicate these gene silencing
43 pathways are likely functional. Strikingly, the Piwi-interacting RNA (piRNA) pathway
44 was absent in *D. gallinae*. In addition, we demonstrate that feeding *Dg vATPase A*
45 dsRNA to adult female *D. gallinae* results in silencing of the targeted gene compared
46 to control mites fed non-specific *lacZ* dsRNA. In *D. gallinae*, dsRNA mediated gene
47 knockdown is rapid, detectable 24 hours after oral delivery of dsRNA and persisted for
48 at least 120 hours.
49

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50 **Conclusions:** This study has shown the presence of core RNAi machinery
51 components in the *D. gallinae* genome. In addition, we have developed a robust RNAi
52 methodology for targeting genes in *D. gallinae*, which will be of value for studying
53 genes of unknown function and validating potential control targets in *D. gallinae*.

54

55 **Keywords**

56 RNA interference; poultry red mite; functional genomics; gene silencing; gene
57 knockdown

58

59 **Background**

60 Poultry red mite (*Dermanyssus gallinae* (De Geer, 1778)), is an avian
61 haematophagous ectoparasite with a worldwide distribution and a prevalence of 83%
62 in European hen egg laying facilities (1). There are five life cycle stages in *D. gallinae*:
63 egg, larvae, protonymph, deutonymph, and adult; and blood feeding is only a feature
64 of the latter three stages (2, 3). This parasite lives off-host in the cracks and crevices
65 in the hen facilities and only emerges to bite the host in darkness for a bloodmeal,
66 which takes around 30 to 90 min during which each mite consumes ~200 µg of blood
67 per feed (3, 4). Thus, in severe infestations where each laying hen may be infested
68 with up to 500,000 mites, infestation can lead to multiple behavioural and physiological
69 changes in the birds, such as restlessness, irritation, anaemia, feather pecking,
70 cannibalism, and increased mortality rates (5, 6). Also, *D. gallinae* has been reported
71 as the vector for a number of bacterial and viral diseases of birds as well as zoonotic
72 agents (7, 8). Apart from the hen welfare issues caused by *D. gallinae*, it also increases
73 the operational expenditure for hen egg production through losses in feed conversion
74 ratio, downgrading of eggs and decreased egg output (6, 9, 10). Overall, the estimated

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75 annual cost of *D. gallinae* (production loss plus costs of control) was estimated as €231
76 million in Europe in 2017 (6). Conventional control of *D. gallinae* is through the use of
77 chemical acaricide treatments of poultry houses or through systemic acaricides
78 administered via drinking water (11). However, with the increased incidence of
79 resistance against some acaricides (12) and concerns over residues in food, multiple
80 chemical treatments have been withdrawn from use in the EU (2).

81 Development of novel stratergies for control of *D. gallinae* is a priority and gene
82 target identification for development of novel control approaches is facilitated by the
83 recent publication of transcriptomes and the genome of *D. gallinae* (13, 14). One key
84 tool for exploiting these genomic and transcriptomic resources for novel target
85 identification is transcriptional silencing. Since the discovery of RNA interference
86 (RNAi) as a tool for silencing gene expression in the free living nematode,
87 *Caenorhabditis elegans* (15), its application has been widened into various fields
88 including novel arthropod control strategies (16, 17). Irrespective of the organism in
89 which transcriptional silencing is to be investigated, two essential components are
90 required for successful RNAi: 1) the presence of a functional RNAi pathway and 2) an
91 appropriate delivery method for the gene-specific double-stranded RNA (dsRNA) to
92 initiate the silencing process. RNAi pathways are present in many of the mite species
93 investigated to date (reviewed in (18)) and, for several species, delivery of dsRNA has
94 been achieved through soaking mites in solutions containing the dsRNA (e.g. see (19,
95 20)). While the immersion method of dsRNA delivery has also caused gene
96 knockdown in *D. gallinae* (21), high mortality rates were observed, thus hampering
97 interpretation of transcriptional silencing data and necessitating the development of a
98 better approach of dsRNA administration to *D. gallinae*.

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99 Here, we use the recently published *D. gallinae* genome (13) and associated
100 transcriptomic data to describe the RNAi pathway in *D. gallinae* and also investigate
101 an optimised delivery method to ascertain the optimal properties of the dsRNA for
102 RNAi in this species. RNAi mediated gene silencing was investigated in adult female
103 *D. gallinae* mites by targetting *D. gallinae vacuolar ATPase subunit A (Dg vATPase*
104 *A)*, which has previously been targeted in arthropods, including the two spotted spider
105 mite, *Tetranychus urticae* (22).

106

107 ## Methods

108 ### Ethics approval

109 All procedures to gather samples from animals were approved by the Moredun Animal
110 Welfare and Ethical Review Body (AWERB) and were conducted under the legislation
111 of a UK Home Office License (reference PPL 60/03899) in accordance with the
112 Animals (Scientific Procedures) Act of 1986.

113

114 ### RNAi Pathway Gene Annotation

115 Core RNAi pathway components were identified in the *D. gallinae* genome by
116 sequence similarity to RNAi pathway genes from *Drosophila melanogaster*. Twelve
117 core RNAi pathway genes were selected from *D. melanogaster* that included
118 Argonaute proteins, RNaseIII proteins and dsRNA binding proteins. Core *D.*
119 *melanogaster* RNAi components were selected to represent three RNAi pathways,
120 including: miRNA pathway (*argonaute1*, *Dicer 1*, *loquacious*, *Drosha*, *Pasha*, *Exportin-*
121 *5*); siRNA pathway (*Argonaute2*, *Dicer 2*, *R2D2*) and piRNA pathway (*Aubergine*, *Piwi*,
122 *Argonaute3*). Amino acid sequences for all twelve *D. melanogaster* RNAi pathway
123 components were retreived from Flybase (23) and used as query for blastp searches

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124 against predicted peptides from the *D. gallinae* genome (13). Identified *D. gallinae*
125 RNAi components were considered sequence orthologues for a given RNAi pathway
126 component when they met the criteria of reciprocal best hit and included one-one and
127 one-many sequence orthologues. Functional domains were identified in retrieved *D.*
128 *gallinae* sequences using Pfam sequence searches (24).
129

130 **Phylogenetic analysis**

131 For phylogenetic analysis selected argonaute-like protein sequences were aligned
132 using MUSCLE (25). Short sequences (<50% of the protein's consensus length) and
133 predicted non-functional sequences due to absence of either PAZ domain (PF02170)
134 or Piwi domain (PF02171) were removed from the alignment. All sequences used for
135 phylogenetic reconstruction are shown in Additional file 1: Table S1. Ambiguously
136 aligned positions were excluded by trimAL v1.2 (26) and a maximum-likelihood (ML)
137 phylogenetic tree was constructed in MEGA 10.1.8 (27) using a LG+G substitution
138 model. Statistical tree robustness was assessed using bootstrap analysis (1000
139 bootstrap replicates).
140

141 **Amplification of *D. gallinae vATPase A* gene for RNAi validation.**

142 The full-length sequence of *D. gallinae vacuolar ATPase subunit A* (*Dg vATPase A:*
143 DEGAL4806g00010) was retrieved from the OrcAE database for *D. gallinae* (13, 28).
144 To validate the OrcAE gene model, *Dg vATPase A* (DEGAL4806g00010) was used in
145 a blastx search against NCBI nr protein database and sequences with high similarity
146 were retrieved from: *Varroa destructor* (XP_022670783 and XP_022670784);
147 *Tropilaelaps mercedesae* (OQR76956); *Galendromus occidentalis* (XP_003741079)
148 and *Ixodes scapularis* (XP_029849202). All sequences were aligned using MUSCLE

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149 (25) and primers were designed based on conserved regions across *Dg vATPase A*
150 and all other aligned sequences. The clustalx alignment and region used for primer
151 design is shown in Additional file 2: Fig S1. The full-length coding sequence of *Dg*
152 *vATPase A* was amplified using female *D. gallinae* cDNA as template and verified by
153 Sanger sequencing.
154

155 **dsRNA Synthesis**

156 Region 1 (R1: 495 bp, corresponding to exon 4 – 7) and Region 2 (R2: 385 bp,
157 corresponding to exon 8) of the *Dg vATPase A* gene were amplified from cDNA
158 generated from adult female *D. gallinae* using Phusion proof-reading polymerase
159 (Thermo Fisher Scientific, Waltham, MA, USA). Each forward and reverse primer
160 contained an NcoI and NheI restriction enzyme site, respectively, to allow directional
161 cloning into the RNAi vector pL4440 (pL4440 was a gift from Andrew Fire [Addgene
162 plasmid # 1654; http://n2t.net/addgene:1654 ; RRID:Addgene_1654]). Primer
163 sequences are shown in Additional file 3: Table S2. Amplification products for *Dg*
164 *vATPase A* R1 (495 bp) and R2 (385 bp) were digested with NcoI and NheI and cloned
165 into the corresponding restriction enzyme sites of pL4440. Plasmids were used to
166 transform chemically competent *E. coli* JM109 cells (Promega, Madison, WI, USA)
167 and plasmid was isolated *E. coli* transformants using a Wizard® *Plus* SV Minipreps
168 DNA Purification System (Promega, Madison, WI, USA). Both RNAi constructs
169 containing *Dg vATPase A* R1 and R2 were verified by Sanger sequencing. For control
170 (non-target) dsRNA production we used a previously generated construct containing
171 a region of the *E. coli* strain K-12 *lacZ* gene NC_000913 (319bp; 63 – 381 bp of the
172 CDS) cloned into SacI and SmaI sites of pL4440 (19). dsRNA was synthesized using
173 the T7 RiboMAX Express RNAi System (Promega, Madison, WI, USA), according to

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174 the manufacturer's instructions. For RNA synthesis *Dg vATPase A* pL4440 plasmids
175 were linearized with either NcoI or NheI for sense or antisense transcription,
176 respectively. Control *lacZ* pL4440 plasmid was linearized with SmaI or BglII for sense
177 or antisense transcription, respectively. Purified dsRNA was quantified on a NanoDrop
178 One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and analysed
179 by agarose/TAE gel electrophoresis to confirm quality and predicted size.
180
181 **siRNA Synthesis**
182 *Dg vATPase A* siRNAs were synthesised by either *in vitro* digestion of long dsRNAs
183 (Method-1) or chemical synthesis of 27-mer blunt dsRNAs (Method-2). Method-1:
184 Long dsRNAs for R1 and R2 of the *Dg vATPase A* gene and *lacZ* control gene (120
185 µg of each dsRNA) were incubated with 0.2 units/µL ShortCut® RNase III (New
186 England BioLabs, Ipswich, MA, USA) for 3 h at 37°C to produce a heterogeneous mix
187 of short (18-25 bp) siRNA. Reactions were stopped with EDTA according to the
188 manufacturer's protocol, ethanol-precipitated and the size-distribution of digested
189 RNAs validated by electrophoresis using a 4% agarose gel. Method-2: Dicer-substrate
190 siRNAs (27-mer blunt dsRNAs) were designed based on the coding sequence of the
191 *Dg vATPase A* gene and *lacZ* control gene using the Eurofins Genomics siMAX siRNA
192 design tool and commercially synthesized and annealed by Eurofins Genomics
193 (Eurofins Genomics, Ebersberg, Germany). The sequence of each siRNA is shown in
194 Additional file 4: Fig S2.
195
196 **RNAi Feeding Trials**
197 Mixed stage and mixed sex *D. gallinae* mites were collected from commercial egg-
198 laying facilities and stored in vented 75 cm^2 tissue culture flasks (Corning Inc, Corning,

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199 NY, USA) at room temperature for seven days, after which they were stored at 4°C for
200 3 weeks without access to food. For oral delivery of dsRNA and siRNA to *D. gallinae*
201 mites, approx. 100 mites were housed in an *in vitro* feeding device constructed as
202 described previously (29). Each replicate feeding device contained 200 µl of freshly-
203 collected heparinised goose blood (20 units/ml) with dsRNA at concentrations
204 described in each experiment. For each dsRNA feeding experiment, biological
205 replicates consisted of independent group of mites in replicate feeding devices (n = 3
206 - 6 depending on experiment). Feeding devices were placed in a Sanyo MLR-351H
207 relative humidity incubator for 3h at 39°C; 21h at 25°C both at 85% relative humidity.
208 After 24 h, fed adult female mites were recovered from each replicate feeding divice
209 and transferred to separate labelled 1.5ml tubes, which were held at 25°C in dark
210 conditions for the duration of the experiment. Mites from each replicate group were
211 flash-frozen in liquid nitrogen at time-points indicated in each experiment and stored
212 at -70°C for later RNA extraction.
213
214 **Quantitative real-time PCR (qPCR) analysis**
215 Real-time quantitative polymerase chain reaction (qPCR) was used to quantify *Dg*
216 *vATPase A* gene expression in adult female mites from RNAi feeding trials. Mites were
217 homogenized with a tube pestle and total RNA was isolated using an RNeasy® plus
218 micro kit (Qiagen, Hilden, Germany) which included a gDNA eliminator spin-column.
219 Total RNA was quantified using a NanoDrop One (Thermo Fisher Scientific, Waltham,
220 MA, USA) and first-strand cDNA synthesized using a QuantiTect® Reverse
221 Transcription Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol.
222 qPCR primers were designed using Primer3Plus (30), primer sequences are
223 shown in Additional file 3: Table S2. Primers were checked for specificity by alignment

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224 of *D. gallinae* target sequences with goose *Anser cygnoides v-ATPase A*
225 (XM_013196364) and *GAPDH* (XM_013199522). In addition, qPCR products were
226 sequenced demonstrating amplification of only *Dg vATPase A* and *D. gallinae*
227 *GAPDH*. For construction of standard curves, qPCR primers were used to amplify *Dg*
228 *vATPase A* (DEGAL4806g00010) and *GAPDH* (DEGAL4146g00090) from adult
229 female *D. gallinae* cDNA. Amplification products were cloned into pJET1.2 (Thermo
230 Fisher Scientific, Waltham, MA, USA) and verified by DNA sequencing. Plasmids were
231 used in qPCR experiments to construct standard curves from 10^2–10^8 copies of each
232 gene. Ten microlitre qPCR reactions comprised 1× PowerUp SYBR green master mix
233 (Thermo Fisher Scientific, Waltham, MA, USA), 500 nM of forward and reverse
234 primers, and cDNA derived from 1 ng total RNA for each sample. PCR reactions were
235 performed on an Applied Biosystems 7500 Real Time PCR System; thermal cycling
236 conditions were 50°C for 2 min, 95°C for 2 min, followed by 40 cycles at 95°C for 15
237 s, 58°C for 15 s, and 72 °C for 1 min. *Dg vATPase A* gene expression was normalized
238 to housekeeping gene *GAPDH* and expression levels reported relative to control (*lacZ*)
239 dsRNA fed mites. qPCR experiments were performed in triplicate and included no
240 template controls and no reverse transcription controls with each run.
241

242 **Statistical analyses**

243 Analysis of *Dg vATPase A* gene expression levels in RNAi feeding trials were
244 performed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, La
245 Jolla, CA, USA). Datasets were analysed using either Student's *t-test* or one-way
246 ANOVA with Dunnett's multiple comparison test (as indicated) and p-values of <0.05
247 were considered significant.
248

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249 ## Results

250 ### miRNA and siRNA pathways are present in the *D. gallinae* genome

251 We used a systematic search for core RNA interference (RNAi) genes involved in the
252 siRNA pathway (*Argonaute2*, *Dicer 2*, *R2D2*); miRNA pathway (*argonaute1*, *Dicer 1*,
253 *loquacious*, *Drosha*, *Pasha*, *Exportin-5*); and piRNA pathway (*Aubergine*, *Piwi*,
254 *Argonaute3*) in *D. gallinae*. Our searches identified *D. gallinae* orthologues of at least
255 one core gene in the siRNA and miRNA and pathways (See both Fig 1 and Table 1)
256 and did not identify piRNA pathway orthologues, suggesting that this pathway is not
257 present in *D. gallinae*.

258

259 ### siRNA Pathway genes

260 Of the three currently known core siRNA pathway genes from *Drosophila* (*Argonaute2*,
261 *Dicer2* and *R2D2*), we identified an orthologue of *Dicer2* in *D. gallinae*
262 (DEGAL2576g00010; [Table 1]) which is required to cleave and yield a mature siRNA.
263 Domain analysis of *D. gallinae* Dcr-2 revealed a similar domain architecture to the
264 well-characterised *Drosophila* Dcr-2, although the PAZ domain was absent in *D.*
265 *gallinae* Dcr-2 (Additional file 5: Fig S3). Gene expression data available through
266 OrcAE shows that *D. gallinae Dcr-2* is universally expressed in adult male and female
267 mites and all other life-stages. Our searches did not identify any other core siRNA
268 pathway genes in *D. gallinae* and, while our searches identified a large Ago family
269 (containing 25 members), it did not include a *D. gallinae Ago2* orthologue.

270

271 ### miRNA Pathway genes

272 We identified orthologues of all six core miRNA pathway genes in *D. gallinae*.
273 Identified *D. gallinae* miRNA pathway genes included: <u>*Drosha*</u> (DEGAL3563g00160;

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274 [Table 1]) and *Pasha* (DEGAL6243g00040; [Table 1]) required for miRNA
275 biosynthesis. *Exportin-5* (DEGAL4407g00370; [Table 1]) required for export of pre-
276 miRNA from the nucleus to cytoplasm. *Dicer-1* (DEGAL4207g00210; [Table 1]) and
277 two copies of its binding partner *Loquacious* (DEGAL6165g00020;
278 DEGAL6165g00030; [Table 1]) required to cleave and yield a mature miRNA.
279 *Argonaute-1* (DEGAL5747g00010; DEGAL5147g00020; [Table 1]) required to target
280 and slice complementary RNA transcripts.

281 Domain analysis of *D. gallinae* Dcr-1 (Additional file 5: Fig S3) and *Argonaute-*
282 *1* orthologues (Additional file 6: Fig S4.) demonstrate the presence of functional
283 domains associated with activity. In addition, gene expression data available through
284 OrcAE shows that all identified *D. gallinae* miRNA components are universally
285 expressed in adult male and female mites and all other life-stages.
286

287 **Piwi and Ago2 proteins are absent in the *D. gallinae* genome**

288 *D. gallinae* Ago/Piwi coding sequences were identified based on similarity to two Ago
289 proteins (Ago1 and Ago2) and three Piwi proteins (aub, piwi, Ago3) from *Drosophila*.
290 All Ago/Piwi sequence orthologues identified in *D. gallinae* met the criteria of reciprocal
291 best-hit in *D. gallinae* and *Drosophila* genomes. Using this search methodology we
292 identified 25 Ago orthologues in the *D. gallinae* genome, while no Piwi orthologues
293 were identified (Table 1). Additional searches were performed, using the same search
294 criteria, with Ago/Piwi protein coding sequences from *T. urticae*, a related mite species
295 with a high-quality annotated genome (31). These additional searches did not identify
296 any additional Ago orthologues in *D. gallinae*, and again failed to identify any Piwi
297 coding sequences.

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298 All functional Ago proteins contain two common structural features: PAZ
299 domain (responsible for small RNA binding) and Piwi domain (responsible for catalytic
300 activities) (32). Therefore, in order to identify potential functional *D. gallinae* Ago
301 proteins, all 25 *D. gallinae* Ago orthologues were analysed for domains using the Pfam
302 database (24). These searches identified 16 *D. gallinae* Ago orthologues that
303 contained both PAZ domain (Pfam, PF02170) and Piwi domain (Pfam, PF02171)
304 (Additional file 6: Fig S4). In addition, all 16 *D. gallinae* Ago orthologues have a DEDH
305 catalytic slicer motif within each Piwi domain indicating that these Agos likely retain
306 slicer activity (33).

307 *D. gallinae* Ago orthologues with both PAZ and Piwi domains (a total of 16 *D.*
308 *gallinae* Agos) were compared with orthologues from other arthropods including: the
309 two-spotted spider mite, *T. urticae* (6 Ago orthologues and 7 Piwi orthologues) and
310 insects (as detailed in Fig. 2). Our phylogenetic analysis identified two *D. gallinae* Ago1
311 orthologues (DEGAL5147g00020; DEGAL5747g00010) likely to be involved in the
312 miRNA pathway. The remaining *D. gallinae* Ago orthologues belong to two major
313 clades, the first containing seven members and are closely related to Ago1 proteins
314 (Fig. 2, Ago1-like clade). The second major clade contains seven members and is
315 unique to *D. gallinae* (Fig. 2, Dg Ago clade). Members of the Dg Ago clade show
316 evidence of duplication, with four members (DEGAL3253g00060;
317 DEGAL3253g00070; DEGAL3253g00080; DEGAL3253g00100) present as a tandem
318 array on the same genomic scaffold. Strikingly, none of the identified *D. gallinae* Agos
319 belong to either the Ago2 clade or Piwi clade (Fig. 2).

320

321 **RNA-dependent RNA Polymerase (RdRP) is present in *D. gallinae* genome**

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322 RNAi is a conserved gene silencing mechanism in eukaryotes. In non-animal
323 eukaryotes RNAi mediated gene silencing requires RNA-dependent RNA polymerase
324 (RdRP) proteins (34). However, in the animals investigated thus far, only *C. elegans*
325 requires RdRP for RNAi mediated gene silencing (34). Using *C. elegans* RdRP protein
326 sequence as a query in blastp search against *D. gallinae* we identified 9 RdRP
327 orthologues in the *D. gallinae* genome, that meet the criteria of reciprocal best hit in
328 *D. gallinae* and *C. elegans* genomes (Table 1). Based on gene expression data
329 available through OrcAE, all 9 *D. gallinae* RdRP orthologues are expressed and
330 therefore may play a role in amplification and propagation of silencing signals (Table
331 1).
332

333 **Functional RNAi gene silencing in *D. gallinae***

334 Two regions of the *Dg vATPase A* gene were selected for synthesis of dsRNA. Region
335 1 (R1: 495 bp, corresponding to exon 4 – 7) and Region 2 (R2: 385 bp, corresponding
336 to exon 8) were used for *in vitro* synthesis of dsRNA (Fig. 3). dsRNAs were
337 incorporated into goose blood, which was delivered to adult female *D. gallinae* mites
338 using an *in vitro* feeding device. After feeding, engorged mites were selected based
339 on a visible blood meal contained within the abdomen and were used for gene
340 expression analysis. Mites with no visible signs of feeding were discarded and not
341 used for expression analyses. Feeding R1 and R2 dsRNA (both at 100 ng/μl) in
342 separate feeding trials to adult female *D. galline* mites resulted in a significant 1.9-fold
343 reduction (for both R1 and R2 feeding trials) in expression of *Dg vATPase A* compared
344 with control mites that fed on blood containing non-specific *lacZ* dsRNA [$P<0.05$, one-
345 way ANOVA with Dunnett's multiple comparison test] (Fig. 4a). Under similar feeding
346 methodology, combining R1 and R2 *Dg vATPase A* (100 ng/μl dsRNA, consisting of

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347 an equimolar mix of R1 and R2 dsRNA) dsRNAs resulted in a significant 2.6-fold
348 reduction in expression of *Dg vATPase A* compared with control mites that fed from
349 non-specific *lacZ* dsRNA [$P<0.05$, Student's t-test] (Fig. 4b). During both feeding trials
350 there was no gross observable phenotypic difference between mites fed with *Dg*
351 *vATPase A* dsRNA and mites fed with control *lacZ* dsRNA.
352
353 **RNAi mediated gene silencing in *D. gallinae* is initiated quickly and is long**
354 **lasting**
355 Feeding combined R1 and R2 *Dg vATPase A* dsRNA to adult female *D. gallinae* mites
356 resulted in rapid reduction of *Dg vATPase A* gene expression compared to control
357 mites treated with the non-specific *lacZ* dsRNA. A significant reduction in *Dg vATPase*
358 *A* expression was detectable by 24h post dsRNA delivery, and was maintained for at
359 least 120h post dsRNA delivery (Fig. 5). At each time-point analysed, including 24h,
360 48h, 72h and 120h expression of *Dg vATPase A* was significantly reduced by 2-fold,
361 3.7-fold, 2.9-fold and 3.4-fold, respectively, relative to control (lacZ) fed mites [$P<0.05$,
362 one-way ANOVA with Dunnett's multiple comparison test] (Fig. 5).
363
364 **Oral delivery of siRNAs does not down-regulate target gene expression**
365 Short dsRNAs were produced by either *in vitro* dicer treatment of synthesized long R1
366 and R2 dsRNAs (Method-1) or commercial synthesis of two 27 bp dsRNA
367 corresponding to regions of the *Dg vATPase A* gene (Method-2). The sequence of
368 each synthesized siRNA is shown in Additional file 4: Fig S2. Feeding trials were used
369 to deliver either diced-R1/R2 dsRNAs (Fig. 6a) or synthesized 27 bp dsRNAs (Fig.
370 6b), along with the appropriate control dsRNA to adult female *D. gallinae* mites.
371 Neither diced R1/R2 dsRNAs nor synthesized 27 bp dsRNAs resulted in down

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372 regulation of the *Dg vATPase A* gene expression, and treated mites had comparable
373 expression levels with control fed mites (*P*=0.1073 for diced R1/R2 dsRNAs; *P*=0.6225
374 for synthesized 27 bp dsRNAs trial; Student's t-test).
375

376 **Discussion**

377 Here, we demonstrate the presence of genes encoding components of two of the main
378 RNAi pathways in the genome of *D. gallinae*, namely the siRNA and miRNA pathways.
379 Both exogenous and endogenous siRNA pathway components were identified in the
380 *D. gallinae* genome but the major piRNA components, *Piwi*, *Aub* and *Ago3* genes,
381 were lacking indicating either very dissimilar sequences of these genes or, more
382 probable, the absence of this pathway in *D. gallinae*. We also demonstrate the utility
383 of an *in vitro* feeding methodology (29) for the successful delivery of dsRNA to initiate
384 prolonged, gene-specific RNAi in *D. gallinae* but show that orally-delivered short
385 siRNA failed to initiate RNAi for the gene investigated herein.

386 In arthropods, three main RNAi mechanisms co-exist (small interfering RNAs
387 (siRNA), microRNAs (miRNA), and Piwi-interacting RNAs (piRNA) (17) but in several
388 species of the Acari investigated to date, the piRNA pathway has been notably absent.
389 The piRNA pathway appears to be absent in: the sheep scab mite, *Psoroptes ovis*
390 (19), the human itch mite, *Sarcoptes scabiei* (18) and the house dust
391 mites, *Dermatophagoides farinae* (35), *Dermatophagoides pteronyssinus* (18) and
392 *Euroglyphus maynei* (18). In *Drosophila*, where the piRNAi biology is best
393 characterised, the piRNA pathway silences transposons during germline
394 development, thereby protecting the inherited genome from mutation (36). The
395 apparent lack of the piRNA pathway in *D. gallinae* and other members of the Acari,
396 reflects the dynamic nature of RNAi pathways in mites and may indicate species-

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397 specific biology. The molecular mechanism/s which protect *D. gallinae* and other Acari
398 lacking the piRNA pathway against the deleterious effects of transposon activity in the
399 germline await investigation.

400 Our work presented here demonstrated that components of the siRNA pathway
401 are present in the *D. gallinae* genome. One notable absence was the lack of a *D.*
402 *gallinae* Ago2 orthologue, onto which the mature siRNA is loaded and is required for
403 gene silencing (Fig 1). Although a definitive orthologue of Ago2 was not discovered in
404 *D. gallinae*, the argonaute family was expanded with 25 family members, some of
405 which were unique to *D. gallinae*, raising the possibility that, in *D. gallinae, Ago2* is
406 replaced by another yet uncharacterised argonaute. Our experimental work presented
407 here confirms that although an Ago2 orthologue is missing in *D. gallinae*, the siRNA
408 pathway is functional and can be exploited be feeding exogenous dsRNA, resulting in
409 specific knockdown of the targeted *Dg vATPase A* gene. In our RNAi feeding
410 experiments, *Dg vATPase A* was chosen as a target, as it has been previously
411 targetted in *T. urticae*, where down-regulation resulted in a colour change phenotype
412 in *vATPaseA* gene silenced *T. urticae* mites (22). Using a similar dsRNA feeding
413 methodology, we achieved gene silencing of *Dg vATPase A*, but this was not
414 associated with any notable phenotypic changes. In experiments investigating dsRNA
415 length, we were able to achieve robust and reproducible *Dg vATPase A* knockdown
416 using dsRNAs of 385bp and 495bp. However, when we targeted the same gene with
417 short siRNAs, produced from either dicing the long dsRNAs or commercially
418 synthesizing a short 27bp siRNA, the gene knockdown effect was lost. Recent RNAi
419 experiments in *T. urticae* demonstrate there is a size threshold for effective gene
420 silencing, where long dsRNAs resulted in robust gene silencing, while shorter dsRNAs
421 (100 – 200 bp) were not effective (37). In *D. gallinae* the lower limit of dsRNA size for

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422 efficient gene silencing is currently unknown and awaits further investigation. The lack
423 of gene silencing by short siRNAs in *D. gallinae* may result from three possibilities: i)
424 uptake of short dsRNA from food is limited and therefore does not induce RNAi
425 pathway; ii) the abundance of siRNAs saturates the intracellular RNA uptake system
426 system; or iii) short dsRNAs are transported but not recognized by cellular RNAi
427 processing machinery.

428 Functional RNAi is an important tool in both model and non-model organisms
429 to investigate genes of unknown function. For example, RNAi has been exploited in
430 *Drosophila* to investigate gene function in cultured cells using high throughput
431 screening methods (38). Functional RNAi in *D. gallinae*, using methodologies
432 described here, is particularly timely for investigating genes of unknown function in *D.*
433 *gallinae*. Recent completion of the draft *D. gallinae* genome sequence (959 Mbp
434 assembly) identified 14,608 protein coding genes, of which 768 appear to be unique
435 to *D. gallinae*, without similarity to proteins in the NCBI nr protein database (13).
436 Therefore development of a robust and repoducible RNAi methodology, coupled with
437 -omic technologies offers a powerful approach to begin investigating gene function
438 and biology that is unique to *D. gallinae*. In addition, RNAi methodologies for *D.*
439 *gallinae* are likely to be a useful tool in the development of novel control methods,
440 including vaccine development, as it will support the research community with interests
441 in *D. gallinae* control (39-41). RNAi mediated gene silencing in *D. gallinae*, coupled
442 with either *in vitro* bioassays (29), or on-bird feeding assays (42) will allow rapid
443 screening of potential *D. gallinae* vaccine candidates and druggable targets.
444 Significantly, utilising RNAi to screen for vaccine candidates in *D. gallinae* will likely
445 speed up the process of antigen discovery and in turn conform to 3R principles of
446 using fewer animals for vaccine trials.

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447

448 ## Conclusions

449 We found evidence for the presence of two RNAi pathways in *D. gallinae* and also
450 successfully demonstrated that our functional gene knockdown protocol can be
451 initiated by feeding gene-specific dsRNA in an improved *in vitro* feeding device. This
452 opens the door for larger scale, genome-wide screening for novel *D. gallinae* control
453 targets and also the opportunity to ascribe functions, using phenotypic assays, to the
454 multiple genes of unknown function identified within the *D. gallinae* genome, for which
455 no homologues exist in other species.

456

457 ## Availability of data and material

458 The *Dg vATPase A* nucleotide coding sequence is available in the NCBI database
459 using the following accession number: MW032475.

460

461 ## Acknowledgements

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468

469 ## Authors' contributions

470 AJN, DRGP, STGB, WC designed the study; DRGP, WC performed research; AJN,
471 DRGP, STGB, WC drafted the paper. ASB, FN, JMS and KB edited the paper. FN and

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472 KB trained DRGP and WC in use of *in vitro* feeding devices. All authors read and
473 approved the final manuscript.
474

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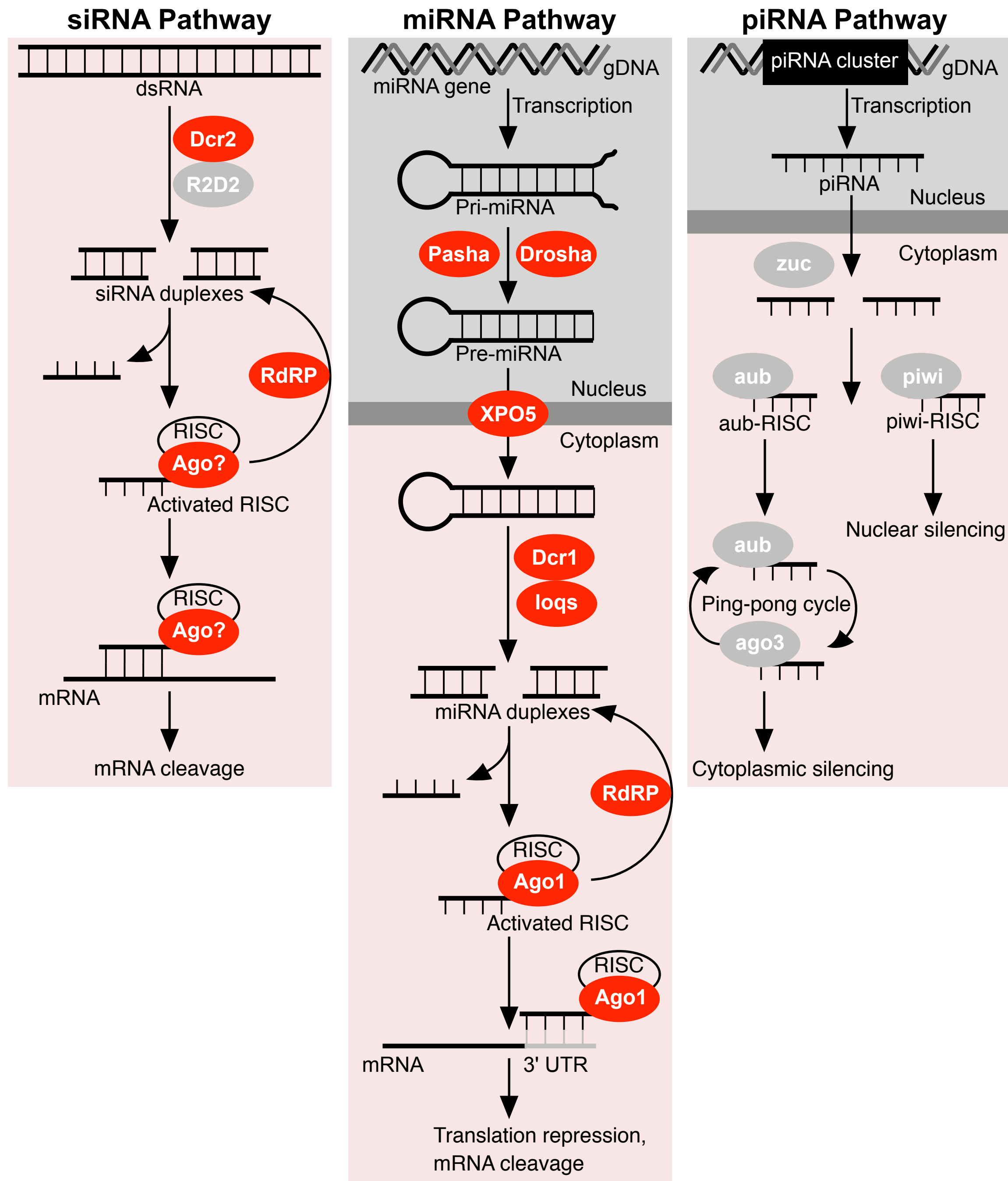
620 **Figures**



621

622 **Fig. 1 RNA interference (RNAi) pathways in *D. gallinae*.** Core RNAi pathway
623 enzymes are shown in either red (present in *D.gallinae*) or grey (absent in *D. gallinae*).
624 **siRNA pathway:** dsRNA (either viral or experimentally introduced) is processed by
625 Dicer-2 (Dcr-2) into 21-23 nt siRNAs and loaded into the RISC complex. Argonaute
626 (Ago) cleaves the passenger strand of the siRNA and retains the guide strand which

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627 guides the active siRISC complex to the target mRNA. Full complementarity between
628 the guide siRNA strand and target mRNA leads to cleavage of the mRNA. **miRNA**
629 **pathway:** The miRNA gene is transcribed in the nucleus to generate pri-miRNA which
630 is then cleaved by Drosha and Pasha to form a pre-miRNA. The pre-miRNA is
631 transported to the cytoplasm through Exportin-5 (XPO5) and cleaved by Dicer-1 (Dcr-
632 1) to yield miRNA. The miRNA is loaded into the RISC complex and argonaute (Ago)
633 cleaves the passenger strand of the miRNA and retains the guide strand which guides
634 the active miRISC complex to the target mRNA. Partial complementarity between the
635 guide strand and target mRNA leads to either translation repression or cleavage of the
636 mRNA. miRNAs usually target several genes, with shared sequences in the 3'
637 untranslated region (UTR). In both pathways siRNAs and miRNAs are amplified by
638 RNA-dependent RNA polyrmerase (RdRP). **piRNA pathway:** The piwi-interacting
639 RNA (piRNA) pathway functions in germline cells to protect against transposable
640 elements. Antisense piRNAs are transcribed from repetitive elements in gDNA and
641 processed by zucchini (zuc) into 26 – 32 nt primary piRNAs. Primary piRNAs associate
642 with either piwi or abuergine (Aub). Piwi associated piRNAs are translocated to the
643 nucleus, while Aub associated piRNAs cleave cytoplasmic transposon transcripts and
644 trigger a 'ping-pong' piRNA amplification. Following transposon transcript cleavage
645 argonaute 3 (AGO3) is loaded with secondary piRNAs which in turn produce piRNAs
646 that associate with Aub, resulting in silencing of cytoplasmic transposon transcripts.

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647

648 **Fig. 2 Phylogenetic analysis of Argonaute-like proteins from *D. gallinae* and**
649 **other arthropods.** All *D. gallinae* sequences are available at OrcAE using DEGAL
650 accession numbers shown in the alignment. The species and accession number of all
651 other sequences are shown in Additional file 1: Table S1. All protein sequences were
652 aligned using MUSCLE and phylogenies reconstructed using maximum-likelihood
653 (ML) methods with a LG+G substitution model. Bootstrap support values >0.6 from
654 1000 replicates are shown at each major node. Scale bar represents 0.3 substitutions
655 per amino acid site. *D. gallinae* Argonaute genes that are located on the same genomic
656 scaffold are indicated by a superscript letter.

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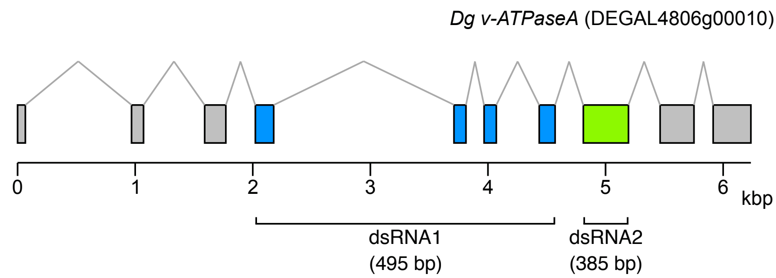

658

659

660 **Fig. 3 Regions of *Dg vATPase A* gene used for dsRNA synthesis.** Schematic
661 representation of the *Dg vATPase A* gDNA locus in *D. gallinae* gDNA scaffold
662 DEGAL4806g00010 (34.1 kbp) location of Region 1 (R1) [exons 4, 5, 6 and 7] and
663 Region 2 (R2) [exon 8] used for dsRNA synthesis.

664

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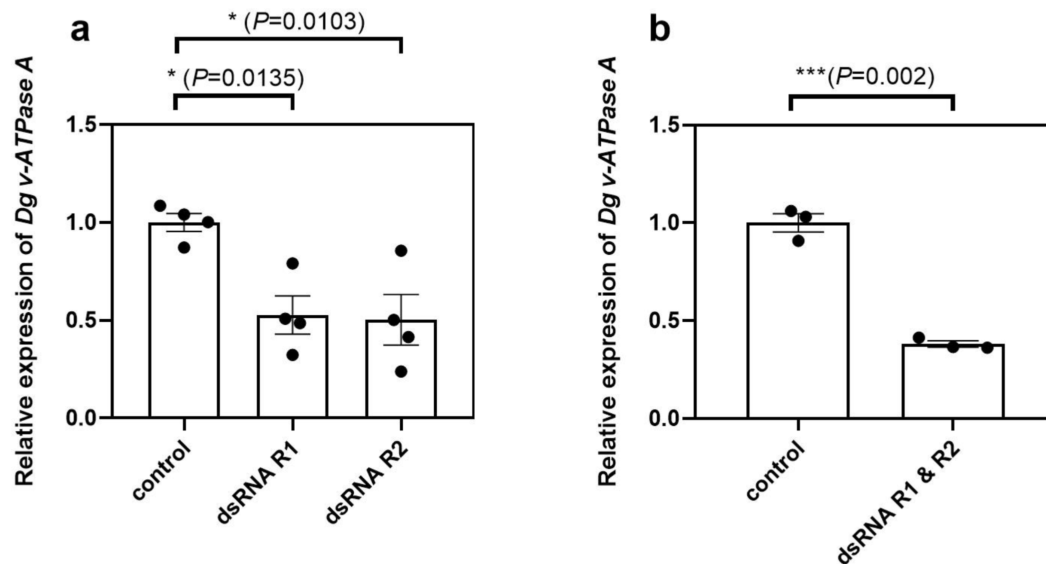

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666 **Fig. 4 RNAi gene knockdown of *Dg vATPase A* in *D. gallinae*.** qPCR gene
667 expression analysis of *Dg vATPase A* expression in adult female *D. gallinae* mites fed
668 on *lacZ* dsRNA (control) or *Dg vATPase A* dsRNA from Region 1 (R1) or Region 2
669 (R2). R1 and R2 *Dg vATPase A* dsRNAs were either fed separately **a** or combined **b**
670 with a total dsRNA at 100 ng/µl (final concentration, with experiment **b** containing
671 equimolar amounts of R1 and R2 dsRNA) in each experiment. *Dg vATPase A*
672 expression shown at 96h post-feed and is normalised to *GAPDH* expression.
673 Individual data points for biological replicates are shown with mean ± SEM indicated
674 (n=3-4). Asterisks represent significant difference ($P<0.05$) between treatments
675 determined by **a** one-way ANOVA with Dunnett's multiple comparison test or **b**
676 Student's t-test.

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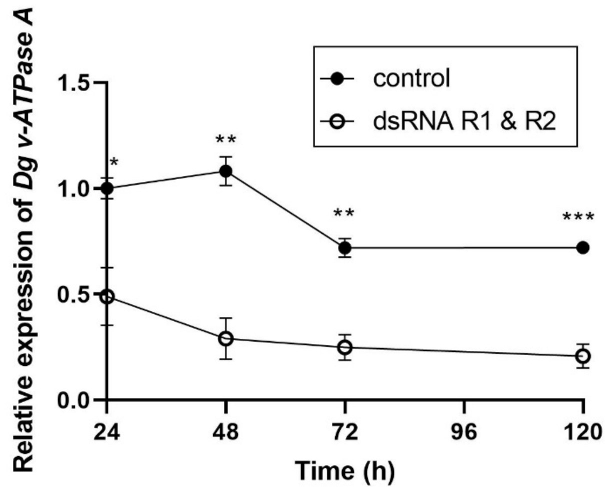

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679 **Fig. 5 Persistence of RNAi gene knockdown of *Dg vATPase A* in *D. gallinae*.**
680 qPCR gene expression analysis of *Dg vATPase A* expression in adult female *D.*
681 *gallinae* mites fed on *lacZ* dsRNA (control) or combined *Dg vATPase A* dsRNA from
682 Regions 1 and 2 (R1 and R2). In control and *Dg vATPase A* dsRNA feeding
683 experiments total dsRNA was delivered at 100 ng/μl (final concentration, containing
684 equimolar amounts of R1 and R2 dsRNA) and *Dg vATPase A* expression levels
685 monitored over a 120h post-feed time course. Individual data points for biological
686 replicates are shown with mean ± SEM indicated (n=3-4). Asterisks represent
687 significant difference (*P*<0.05) between treatments determined by one-way ANOVA
688 with Dunnett's multiple comparison test

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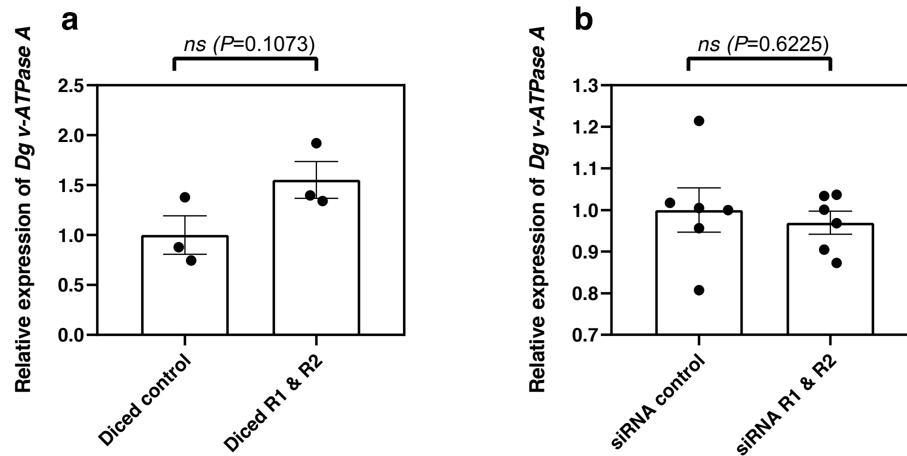

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691 **Fig. 6 siRNAi gene knockdown of *Dg vATPase A* in *D. gallinae*.** qPCR gene
692 expression analysis of *Dg vATPase A* expression in adult female *D. gallinae* mites fed
693 on *Dg vATPase A* siRNAs or *lacZ* siRNA (controul. **a** Long R1 and R2 *Dg vATPase A*
694 dsRNAs and long *lacZ* dsRNA were for diced to produce short siRNAs and fed at 100
695 ng/µl. **b** siRNAs (27-mer) for *Dg vATPaseA* and *LacZ* (control) were commercially
696 synthesized and fed at 100 ng/µl. For both **a** and **b** *Dg vATPase A* expression shown
697 at 96h post-feed and is normalised to *GAPDH* expression. Individual data points for
698 biological replicates are shown with mean ± SEM indicated (n=3-6). No significant
699 differences (ns) were detected between treatment groups using Student's t-test.

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701 **Tables.**

702 **Table 1. Identification of *D. gallinae* core RNAi pathway genes**

Gene name [Gene symbol] (species)	*D. gallinae* gene ID	Blast E-value
Dicer-1 [*Dcr-1*] (D)	DEGAL4207g00210	1.00E-141
Dicer-2 [*Dcr-2*] (D)	DEGAL2576g00010	2.00E-57
Partner of Drosha [*pasha*] (D)	DEGAL6243g00040	1.00E-97
Drosha [*Drosha*] (D)	DEGAL3563g00160	0
Loquacious [*loqs*] (D)	DEGAL6165g00020[f]	2.00E-32
	DEGAL6165g00030[f]	4.00E-14
Argonaute-1, *-2*, *-3* [*AGO1, 2, 3*] (D)	DEGAL5747g00010	0
	DEGAL5147g00020[a]	0
	DEGAL2433g00050	3E-167
	DEGAL1807g00010	2E-146
	DEGAL5891g00010	1E-141
	DEGAL2763g00020	2E-141
	DEGAL2329g00030	3E-138
	DEGAL3376g00010	3E-118
	DEGAL1832g00030	5E-106
	DEGAL3253g00060[b]	2E-101
	DEGAL3253g00070[b]	1E-100
	DEGAL5243g00010	6E-99
	DEGAL3253g00080[b]	9E-99
	DEGAL3253g00100[b]	1E-98
	DEGAL867g00020[c]	4E-98
	DEGAL5130g00010	3E-91

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	DEGAL4347g00070	1E-76
	DEGAL1695g00010[d]	5E-62
	DEGAL1695g00020[d]	3E-56
	DEGAL4104g00020	3E-38
	DEGAL867g00010[c]	3E-37
	DEGAL6462g00020[e]	8E-31
	DEGAL6462g00010[e]	1E-21
	DEGAL2892g00010	2E-20
	DEGAL5147g00040[a]	9E-16
Exportin-5 [*XPO5*] (D)	DEGAL4407g00370	2.00E-25
RNA-dependent RNA polymerase [*RdRP*] (C)	DEGAL1833g00010	1.00E-100
	DEGAL2592g00050	1.00E-100
	DEGAL4182g00030	5.00E-100
	DEGAL2262g00020	2.00E-98
	DEGAL6675g00010	1.00E-94
	DEGAL6621g00090	5.00E-92
	DEGAL6161g00150[g]	2.00E-52
	DEGAL3284g00080	6.00E-25
	DEGAL6161g00170[g]	4.00E-22

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704 Each identified *D. gallinae* gene is an orthologue of *D. melanogaster* (D) or *C. elegans*
705 (C) RNAi pathway genes based on best reciprocal BLAST hit. Genes located on the
706 same *D. gallinae* scaffold are highlighted using the superscript letters a-g.

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708 # Supplementary Figures and Tables

709

710 **Additional file 1: Table S1.**

711 **Ago sequence accession numbers used for phylogenetic reconstruction.**

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```
DEGAL4806g00010      MSLPKISDAKESEYGYVHAVSGPVVTARDMEGAAMYELVRVGHEELVGEIIKLEGGMATI    60
XP_022670784         MSLPKILDAKESEYGYVHAVSGPVITARDMEGAAMYELVRVGHDELVGEIIKLEGGMATI    60
OQR76956             MSLPKISDAKESEYGYVHAVSGPVVTARDMEGAAMYELVRVGHEELVGEIIKLEGGMATI    60
XP_003741079         MSLPKVADAKESDYGYVHAVSGPVVTARDMDGAAMYELVRVGHDELVGEIIKLEGGMATI    60
                     *****: *****:***********:*****:************:****************

DEGAL4806g00010      QVYEETSGVTVGDPVLRTRKPLSVELGPGIMNSIFDGIQRPLRDINALTQSIYIPK----    116
XP_022670784         QVYEETSGVTVGDPVLRTRKPLSVELGPGIMNSIFDGIQRPLREINSKTQSIYIPK----    116
OQR76956             QVYEETSGVTVGDPVLRTRKPLSVELGPGIMNSIFDGIQRPLREINATTESIYIPKVSVI    120
XP_003741079         QVYEETSGVTVGDPVLRTRKPLSVELGPGIMNSIFDGIQRPLRDIGVLTDSIYIPK----    116
                     *******************************************:*.  *:******

DEGAL4806g00010      --------GVNVPALSRSTQWEFTPTNIKIGSHVTGGDIYGTG-----------------    151
XP_022670784         --------GINVPALSRNTLWEFTPANVKIGSHMTGGDIFGFVNENSLINHKIMLDPKAR    168
OQR76956             VTYKRPVMGVNVPALSRHSQWEFTPANIKIGSHMTGGDIFGLVHENSMINHKIMLDPKAR    180
XP_003741079         --------GVNVPALPRDTQWEFNPANIKIGSHMTGGDVFGTVVENSMINHKIMLEPKAR    168
                             *:***** * : ***.*:*:*****:****::*

DEGAL4806g00010      --NAYIAAPGNYTVDDVVLETEFDGEKKKYTMMQIWPVRQPRPTAEKLAANFPLLTGQRV    209
XP_022670784         GTVTFVAAPGNYTVDEVVLETEFDGEKKKYTMMQVWPVRQPRPTAEKLAANHPLLTGQRV    228
OQR76956             GTVTYIAAPGNYTVNDVVLETEFDGEKKKYTMMQVWPVRQPRPTAEKLAANHPLLTGQRV    240
XP_003741079         GTVTYIAAPGNYTVEDVVLETEFDGEKKKYTMLQVWPVRQPRPTAEKLAANYPLLTGQRV    228
                        :::********::****************:*:****************.********

DEGAL4806g00010      LDTLFPCVQGGTTAIPGAFGCGKTVISQSLSKFSNSDAIIYVGCGERGNEMSEVLRDFPE    269
XP_022670784         LDALFPCVQGGTTAIPGAFGCGKTVISQSLSKYSNSDAIIYVGCGERGNEMSEVLRDFPE    288
OQR76956             LDSLFPCVQGGTTAIPGAFGCGKTVISQSLSKFSNSDAIIYVGCGERGNEMSEVLRDFPE    300
XP_003741079         LDSLFPCVQGGTTAIPGAFGCGKTVISQSLSKYSNSDAIIYVGCGERGNEMAEVLRDFPE    288
                     **:*****************************:*****************:********

DEGAL4806g00010      LTTEVNGKQVSIMQRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYNVAMMADSTSR    329
XP_022670784         LTTEVNGQQVSIMQRTALVANTSNMPVAAREASIYTGITLSEYFRDMGFNVAMMADSTSR    348
OQR76956             LTTEVNGRQVSIMQRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYNVAMMADSTSR    360
XP_003741079         LTTEVDGQQVSIMQRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYNVAMMADSTSR    348
                     *****:*:****************************************:***********

DEGAL4806g00010      WAEALREISGRLAEMPADSGYPAYLAARLASFYERAGRVKCLGNPEREGSVTIVGAVSPP    389
XP_022670784         WAEALREISGRLAEMPADSGYPAYLAARLASFYERAGRVKCLGNPEREGSVTIVGAVSPP    408
OQR76956             WAEALREISGRLAEMPADSGYPAYLAARLASFYERAGRVKCLGNPEREGSVTIVGAVSPP    420
XP_003741079         WAEALREISGRLAEMPADSGYPAYLSARLASFYERSGRVKCLGNPEREGSVTIVGAVSPP    408
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DEGAL4806g00010      GGDFSDPVTSATLSIVQVFWGLDKKLAQRKHFPSINWLISYSKYTNALDEYYDKNFSDFT    449
XP_022670784         GGDFSDPVTSATLSIVQVFWGLDKKLAQRKHFPSVNWLISYSKYTNALDEFYDKNFPEFT    468
OQR76956             GGDFSDPVTSATLSIVQVFWGLDKKLAQRKHFPSINCHISYSKYTGALDDYYDKNFPEFT    480
XP_003741079         GGDFSDPVTSATLSIVQVFWGLDKKLAQRKHFPSVNWLISYSKYTNALDEYYDKHYPDFT    468
                     **********************************:*  *******.***::***:: :**

DEGAL4806g00010      QLRNKCSRILQEEEELSEIVQLVGKASLGEREKITLEVAKLLKDDFLQQNGYTPYDRYCP    509
XP_022670784         ALRNKCSRILQEEEELSEIVQLVGKASLGEREKITLEVAKLIKDDFLQQNGYTPYDRYCP    528
OQR76956             SLRNKCSRILQEEEELSEIVQLVGKGSLGEREKITLEVAKLIKDDYLQQNGYTPYDRYCP    540
XP_003741079         ALRNKCQRILQEEEELSEIVQLVGKASLGEREKITLEVAKLIKDDFLQQNGYTPYDRYCP    528
                      *****.******************.***************:***:**************

DEGAL4806g00010      FYKSVAMLRNMLGFYDMACHAVESTQQSENRITWNVIREATGDILYQLSSMKFKNPKDEG    569
XP_022670784         FYKSVAMLRNMLGFYDMACHAVDATQQSENRITWNVIREAMGDILYQLSSMKFKNPTEES    588
OQR76956             FYKSVSMLRNMLGFYDMACHSVEATQQSENRITWNVIREAMGDILYQLSSMKFKNPKDES    600
XP_003741079         FYKSVGMLRNMLGFYDMACHAVDSTQQSENKITWNVIREAMGETLYQLTSMKFKDPKAET    588
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DEGAL4806g00010      KEKILKDFDELFENMQQDKLSGGRVMISAFSVRRPTDPPLWDHRSY          615
XP_022670784         KEKILKDFDELHENMQQAFRNLED----------------------          612
OQR76956             KEKILKDFDDLYENMQQAFRNLED----------------------          624
XP_003741079         QEKILKDYEELYDNMQQAFRNLED----------------------          612
                     :******:::*.:****   .
```

Additional file 2: Fig S1. Alignment of mite vATPase A proteins. Dg vATPase A and closely related vATPase A from mites were aligned using MUSCLE. Conserved regions in all sequences are highlighted (*). Sequences included in the alignment are:

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736 Dg vATPase A (DEGAL4806g00010); and vATPase A from the following mites: *Varroa*
737 *destructor* (XP_022670784); *Tropilaelaps mercedesae* (OQR76956) and
738 *Galendromus occidentalis* (XP_003741079). Conserved regions, against which
739 coding sequence amplification primers were designed are highlighted in red.
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761 **Additional file 3: Table S2. qPCR and RNAi construct primer sequences**

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```
siRNA Region 1

5'- GUC UGA GGU ACU GCG UGA UUU CCC GGA -3'
    ||| ||| ||| ||| ||| ||| ||| ||| |||
3'- CAG ACU CCA UGA CGC ACU AAA GGG CCU -5'


siRNA Region R2

5'- GAA GAA AUA CAC GAU GAU GCA GAU AUG -3'
    ||| ||| ||| ||| ||| ||| ||| ||| |||
3'- CUU CUU UAU GUG CUA CUA CGU CUA UAC -5'


siRNA lacZ control

5'- GGA UUG ACC GUA AUG GGA UAG GUC ACG -3'
    ||| ||| ||| ||| ||| ||| ||| ||| |||
3'- CCU AAC UGG CAU UAC CCU AUC CAG UGC -5'
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787 **Additional file 4: Fig S2. Regions used for synthetic siRNA synthesis.**
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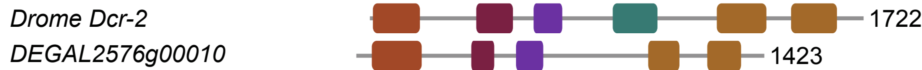
Dicer-1 (Dcr-1)

Dicer-2 (Dcr-2)



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797 **Additional file 5: Fig S3. Domain architecture of *D. gallinae* Dicer proteins.** For
798 comparison the domain architecture of *D. melanogaster* dicer-1 (Dcr-1) and dicer-2
799 (Dcr-2) shown. *D. gallinae* Dcr-1 and Dcr-2 were identified as orthologues of Drome
800 Dcr-1 and Dcr-2, respectively. Pfam (https://pfam.xfam.org) functional domains
801 include: Helicase_C (Helicase conserved C-terminal domain, PF00271); Dicer_dimer
802 (Dicer dimerisation domain, PF03368); PAZ (PAZ domain, PF02170); Ribonuclease_3
803 (Ribonuclease III domain, PF00636); DEAD (DEAD/DEAH box helicase, PF00270).
804 The length of each protein is shown as number of amino acids.

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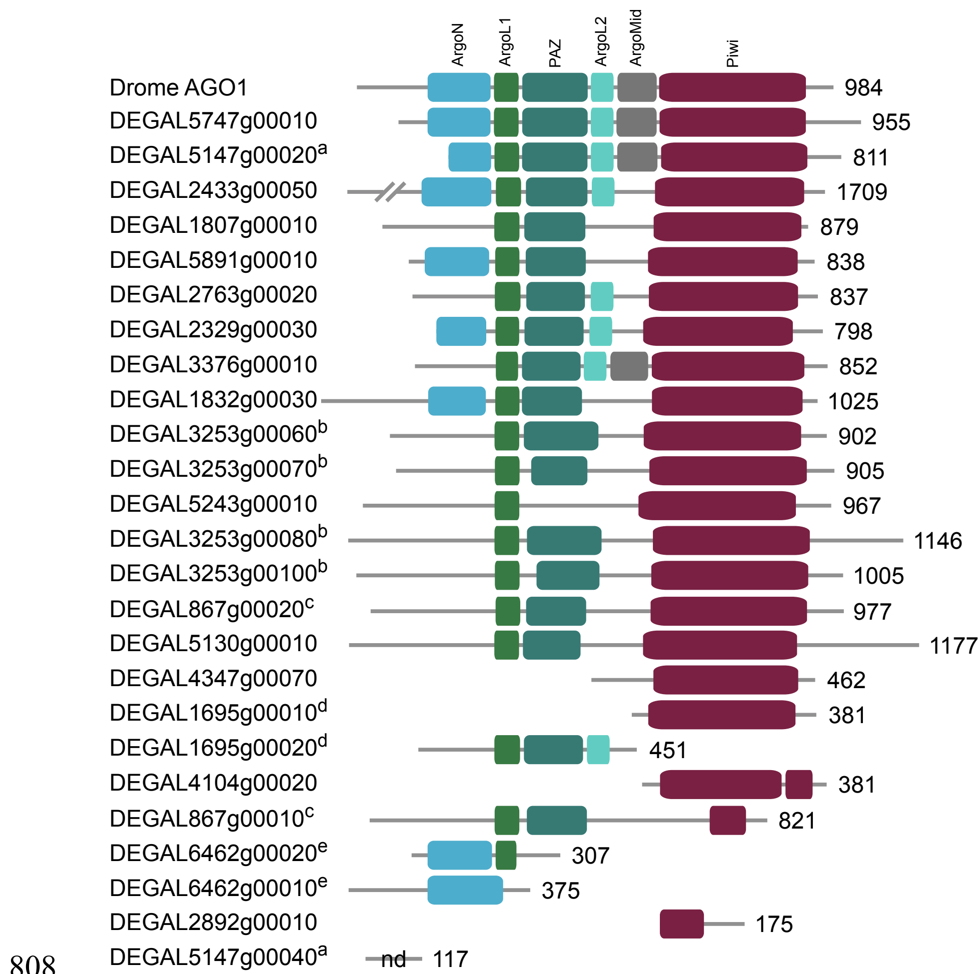

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809 **Additional file 6: Fig S4. Domain architecture of *D. gallinae* argonaute proteins.**

810 For comparison the domain architecture of *D. melanogaster* argonaute-1 (Drome
811 Ago1) is shown. *D. gallinae* argonaute proteins were identified as orthologues of
812 Drome Ago1 and are ranked in order of best blast hit to Drome AGO1. Pfam
813 (https://pfam.xfam.org) functional domains include: ArgoN (N-terminal domain of
814 argonaute, PF16486); ArgoL1 (argonaute linker 1 domain, PF08699); PAZ (PAZ
815 domain, PF02170); ArgoL2 (argonaute linker 2 domain, PF16488); ArgoMid (mid
816 domain of argonaute, PF16487); Piwi (Piwi domain, PF02171). Genes located on the
817 same *D. gallinae* scaffold are highlighted using the superscript letters a-e. The length
818 of each protein is shown as number of amino acids. No Pfam domains were detected
819 (nd, none detected) for DEGAL5147g00040.