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# 1 **IGFBP-rP1, a strongly conserved member of the androgenic hormone** 2 **signalling pathway in Isopoda**

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## 16 **Abstract**

17 The first protein which has been described to interact with the malacostracan Androgenic Gland  
18 Hormone (AGH) is a binding protein called IGFBP-rP1. It has been identified and studied in several  
19 species of decapods, in which its interaction with the masculinizing hormone and its expression  
20 patterns have been established in several ways. However, this protein remains uncharacterised to  
21 date in the other malacostracan orders, like Amphipoda and Isopoda, although they were historically  
22 the first ones in which the androgenic gland and the corresponding hormone were respectively  
23 described. In this article, we identified the IGFBP-rP1 of isopods and established its implication in  
24 the pathway of the AGH with a silencing approach in the model species *Armadillidium vulgare*. We  
25 also showed that this gene is expressed in all the tissues of males and females, with a similar pattern

26 in animals infected with *Wolbachia*, a feminizing endosymbiont of several isopod species. The  
27 expression pattern did not differ during the development of uninfected and infected animals either.  
28 We finally studied the evolution of the IGFBP-rP1 in 68 isopod species, looking for conserved  
29 motifs and evidence of natural selection. Altogether, our results showed that this gene is  
30 constitutively expressed and strongly conserved in isopods, in which it likely constitutes a key  
31 element of the insulin/IGF signalling pathway. However, we also illustrated that IGFBP-rP1 is not  
32 sufficient on its own to explain the different developmental paths taken by the males and the  
33 females or feminized genetic males.

34

### 35 **Keywords (max 6)**

36 *Armadillidium vulgare*

37 Insulin-like Growth Factor Binding Protein-related Protein (IGFBP-rP)

38 Insulin/IGF signalling pathway

39 Isopoda

40 Sexual differentiation

41 *Wolbachia*

42

### 43 **Abbreviations**

44 AGH, androgenic gland hormone; ILP, insulin-like peptide; IAG, insulin-like androgenic gland  
45 hormone; IGFBP, insulin-like growth factor binding protein; IGFBP-rP1, IGFBP-related protein;  
46 ISP, insulin/IGF signalling pathway; SIBD, single insulin-binding domain.

47

## 48 **1. Introduction**

49 In malacostracan crustaceans (e.g. amphipods, decapods and isopods), sexual differentiation  
50 relies on a unique proteinaceous hormone, called the Androgenic Gland Hormone (AGH). The latter,  
51 produced by the so-called androgenic glands, is necessary and sufficient to induce male

52 differentiation of the malacostracan juveniles. This hormone has been studied for several decades,  
53 revealing it belongs to the Insulin-Like Peptide (ILP) Signalling Pathway (ISP), which includes  
54 Insulin-like Growth Factors (IGFs) and insulin-related ILPs like the AGH, also called IAG for  
55 Insulin-like Androgenic Gland hormone (Ventura *et al.*, 2011). Yet, the binding peptides of AGH  
56 remained unknown until a first discovery in a decapod cDNA library in 2013 (Rosen *et al.*, 2013).  
57 This first AGH partner was called Cq-IGFBP (Insulin-like Growth Factor Binding Protein) due to  
58 its belonging to the IGFBP superfamily. In the canonical ISP, these proteins regulate the distribution  
59 and bioavailability of their ligand by sequestering it (Baxter, 2000; Kelley *et al.*, 2002; Bach *et al.*,  
60 2005). This way, they might either inhibit the action of the ligand or promote it by carrying and  
61 releasing it to its target receptors (as a result of various post-translational modifications or upon  
62 interactions with cell surface or matrix) (Forbes *et al.*, 2012). Members of the IGFBP are found  
63 widely in metazoans and are particularly well known in vertebrates, in which they bind IGFs and  
64 not insulin (Hwa *et al.*, 1999). However, using a recombinant Cq-IGFBP, Rosen *et al.* (2013)  
65 proved the specific binding of the AGH (i.e. Cq-IAG) by Cq-IGFBP, confirming the unusual  
66 interaction between this IGFBP and an insulin-related ILP. Moreover, this first description enabled  
67 the subsequent identification of IGFBPs in a variety of other decapod species (Chandler *et al.*, 2015;  
68 Huang *et al.*, 2015, 2016; Li *et al.*, 2015; Ventura-López *et al.*, 2017; Song *et al.*, 2018).  
69 Interestingly, Li *et al.* (2015) and Huang *et al.* (2016) chose to call this protein IAGBP and ILPBP,  
70 respectively, referring to its specific function as a binding protein of the IAG hormone (belonging to  
71 the ILP family, and not IGF). Furthermore, Song *et al.* (2018) referred to this gene as IGFBP7  
72 whereas Huang *et al.* (2015) preferred to call this protein an IGFBP-rP (IGFBP-related protein).  
73 Indeed, structural and phylogenetic studies have brought out the existence of two distinct families  
74 within the IGFBP superfamily (Hwa *et al.*, 1999; Rodgers *et al.*, 2008). The *sensu stricto* IGFBP  
75 (IGFBP1-6) have a high affinity for IGF peptides only (and a low one for insulin) and are found  
76 only in vertebrates. They all display two conserved domains: an insulin binding domain (IB),  
77 followed by a thyroglobulin domain. On the other hand, a variety of proteins (including IGFBP7)



78 have been described in both vertebrates and invertebrates and belong to a family of proteins with a  
79 low affinity for IGF peptides (Oh *et al.*, 1996; Radulović *et al.*, 2015) and a high affinity for insulin  
80 (Yamanaka *et al.*, 1997; Radulović *et al.*, 2015). Contrary to IGFBP1-6, their modular architecture  
81 can display various structural domains (Hwa *et al.*, 1999). Considering the structural, functional and  
82 phylogenetic evidence, members of this IGFBP family have been consensually renamed IGFBP-rP  
83 since 1998 (Baxter *et al.*, 1998). Thus, as pointed out by Huang *et al.* (2015), given its structure  
84 (Rosen *et al.*, 2013) and phylogenetic position (Chandler *et al.*, 2015), Cq-IGFBP is actually a  
85 homologue of IGFBP-rP1 (also previously known as IGFBP7), just like all the crustaceans IGFBPs  
86 identified so far. The IGFBP-rP1 is characterized by an IB domain, followed by a Kazal domain  
87 (protease inhibitor) and an immunoglobulin domain (Ig), all domains also found in the described  
88 decapod IGFBPs. Its belonging to the IGFBP-rP family explains why Cq-IGFBP displays such an  
89 affinity for an insulin-related ILP (Cq-IAG). More recently, the molecular interaction between the  
90 AGH and IGFBP-rP1 was confirmed using a yeast-two hybrid assay (Song *et al.*, 2018). In addition  
91 to the evidence at the molecular level, the interaction between decapod IGFBP-rP1 and the AGH  
92 was also demonstrated at the transcriptional level, using RNAi (Li *et al.*, 2015) and modelled *in*  
93 *silico* (Chandler *et al.*, 2017). Interestingly, Chandler *et al.* (2017) predicted that Sv-IGFBP could  
94 bind not only the AGH but also two other ILPs (ILP1 and ILP2), which remain undiscovered to date  
95 in other malacostracan orders. This characteristic is consistent with the fact that IGFBP-rPs seem to  
96 be involved in varied biological processes in invertebrate species with no AGH (metabolism,  
97 immunity, growth...) (N. Li *et al.*, 2012; Mulenga and Khumthong, 2010; Wang *et al.*, 2015, 2016).  
98 The crustacean IGFBP-rPs, due to their additional interaction with AGH, are however the first ones  
99 potentially involved in sexual differentiation. Yet, the exact function of IGFBP-rP1 is still difficult  
100 to assess.

101 In crustacean isopods, using mostly the common woodlouse *Armadillidium vulgare* as a  
102 model species, the sexual development has been extensively studied since the late 1950's, revealing  
103 the precise anatomical sequence of male gonad differentiation that begins after the third moult

104 following the release of the *larvae* from the female ventral pouch (stage 4) (Suzuki and Yamasaki,  
105 1995). Moreover, male differentiation seems to be especially sensitive and is often altered in  
106 isopods, leading to numerous pathological phenotypes such as various intersex individuals (Legrand  
107 and Juchault, 1986; Rigaud and Juchault, 1998). Notably, some strains of the well-known bacteria  
108 *Wolbachia*, a common endosymbiont of woodlice, can alter the sexual differentiation of their hosts  
109 (Bouchon *et al.*, 1998). These strains, known as feminizing *Wolbachia*, have drawn attention as they  
110 induce the differentiation of genetic male *larvae* into functional females and occasionally into  
111 partially feminized males, called male intersexes. In the functional females induced by the presence  
112 of *Wolbachia*, the androgenic glands do not differentiate so that the AGH is not expressed at all.  
113 Besides, these infected females are refractory to the action of the AGH, as androgenic gland grafts  
114 do not masculinize them. In contrast, the male intersexes become refractory in the course of male  
115 differentiation, which results in mixed sexual characters, in particular the coexistence of  
116 hypertrophied androgenic glands and female genital apertures (Legrand and Juchault, 1986; Rigaud  
117 and Juchault, 1998). In this context, all the molecular partners of the AGH are putative targets of the  
118 feminizing *Wolbachia* (Juchault and Legrand, 1985). This includes IGFBP-rP1, as well as the  
119 transmembrane tyrosine kinase receptors recently described in decapods and now identified in  
120 *A. vulgare* (Herran *et al.*, 2018). Interestingly, some interaction between *Wolbachia* and the ISP has  
121 been described in arthropods (Ikeya *et al.*, 2009; Grönke *et al.*, 2010). To date, the molecular  
122 investigations of the isopod sexual differentiation are however limited to the AGH, so that  
123 subsequent potential alterations in this pathway are not known on the molecular scale either. In this  
124 context, the discovery of IGFBP-rP1 in decapods thus raises several questions relating to its  
125 existence and function in isopods.

126         To tackle these issues, we performed the first characterisation of an isopod IGFBP-rP1 (Av-  
127 IGFBP-rP1). Its affiliation to the IGFBP-rP family was first established by structural analyses. We  
128 then investigated the expression pattern of this gene among tissues of adult males and females and  
129 looked for differential expression during development, in both *Wolbachia*-infected and uninfected

130 lineages of *A. vulgare*. Av-IGFBP-rP1 displayed a broad and constitutive expression, suggesting a  
131 major functional importance. Silencing experiments of the AGH and of the IGFBP-rP1 confirmed  
132 their implication in the physiology of the androgenic glands, as some got hypertrophied following  
133 the inhibition of these genes. The evolutionary conservation of IGFBP-rP1 was thus investigated in  
134 the Isopoda order. Using a transcriptomic approach, as well as RT-PCR and direct Sanger  
135 sequencing, we identified 68 IGFBP-rP1 sequences, one for each of the studied species. Homology  
136 to IGFBP-rP1 was confirmed by phylogenetic reconstructions. Negative selection was further  
137 demonstrated with dN/dS analyses. Altogether, our results showed that this gene is a key element of  
138 the ISP in Isopoda, although not responsible on its own for the different developmental process  
139 between male and female isopods and probably not the target of *Wolbachia*.

140

## 141 **2. Material and methods**

142

### 143 *2.1. Animals*

144 Tissue and developmental expression studies were performed on two lineages of the  
145 common woodlouse (*A. vulgare*) coming from our laboratory rearing: the lineage harvested in  
146 Celles/Belle (France) in 1991, which is infected by the *wVulC Wolbachia* strain and the *Wolbachia*-  
147 free lineage, harvested in Nice (France) in 1967. Tissues from intersexes were collected from  
148 individuals of the *Wolbachia*-infected lineage or intersexes generated from males of the uninfected  
149 lineage that were previously experimentally infected with *Wolbachia* (Juchault and Legrand, 1985;  
150 Rigaud *et al.*, 1991). The silencing experiment was performed with the uninfected lineage only. For  
151 the phylogenetic analysis, we included isopod species for which RNAseq data were already  
152 available and species either sampled from our laboratory rearing or collected from various field  
153 sites in 2015 and 2016. These species represent a wide diversity of ecology (soil-dwelling,  
154 terrestrial, coastal, freshwater and marine species) and mostly a diversity in taxonomic ranks  
155 (Table S1).

156

## 157 2.2. RNA extractions

158 To study the spatial expression of Av-IGFBP-rP1, six to eight *A. vulgare* adults for each sex  
159 (male, female, intersex) and each lineage were dissected in Ringer's solution (394 mM NaCl, 2 mM  
160 KCl, 2 mM CaCl<sub>2</sub>/2H<sub>2</sub>O, 2 mM NaHCO<sub>3</sub>). The following tissues were gradually pooled and frozen  
161 in liquid nitrogen: hemolymph (HE), brain (BR), nerve cord (NC), emptied digestive tract (DT),  
162 digestive *caeca* (CK), muscle/fat (MF), legs (LE) and the different parts of the gonads (ovaries (OV)  
163 in females; androgenic glands (AG), utricles (UC), seminal vesicles (SV), *vasa deferens* (VD) and  
164 sperm (SP) in males). All the dissections were repeated three times independently (four times for  
165 males of the uninfected lineage), requiring around a hundred animals in total. For all the other  
166 analyses (RT-PCR as well as RNAseq and RNAi experiments), whole animals were used. To study  
167 Av-IGFBP-rP1 expression during development, we sampled pools of *larvae* just after birth (i.e.  
168 when they were released from the ventral pouch, week 0) and one, two, three weeks later. From the  
169 fourth week on, juveniles were sampled individually and measured to infer their developmental  
170 stage (4-8) according to Suzuki and Yamasaki (1995). Both dissected tissues or whole animals were  
171 homogenized using a Vibra Cell 75185 sonicator (amplitude of 35%). Total RNA was then extracted  
172 using the RNeasy kit (Qiagen) or TRIzol® reagent and treated with RNase-free DNase I (Qiagen),  
173 according to the manufacturer's protocols. After quantification with NanoDrop™ technology, the  
174 RNAs were stored at -80°C.

175

## 176 2.3. RT-PCR and RT-qPCR

177 Reverse transcriptions were achieved on 500 ng of the previously extracted total RNA using  
178 the SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific) following the supplier's  
179 instructions. Degenerated primers (Sigma-Aldrich) were manually designed using the three  
180 sequences of the IGFBP-rP1 of *A. vulgare*, *A. nasatum* and *Porcellio dilatatus* (Table S2), identified  
181 by TBLASTX in the corresponding public cDNA libraries, using Cq-IGFBP as query. RT-PCR were

182 carried out using Phusion® High-Fidelity DNA Polymerase and annealing temperatures varying  
183 between 45°C and 60°C according to the characteristics of the primers (Table S2), with an  
184 elongation time of 60 s. The amplification products were separated on 1.5% agarose gels and  
185 visualised with ethidium bromide and UV.

186 RT-qPCR were performed using Applied Biosystems™ SYBR™ Green master mixes (5 µL  
187 Sybergreen 5X, 0.5 µL of each primer, 2.5 µL cDNA and sterile water to 10 µL). Primer sequences  
188 are given in Table S2. The reactions were performed on a LightCycler® 480 System (Roche), using  
189 the following program: 95°C for 10 min followed by 45 cycles of (95°C for 10 s, 60°C for 10 s,  
190 72°C for 20 s). Melting curves were established (65°C-97°C) to check the specificity of the PCR  
191 products. The Av-IGFBP-rP1 expression levels were analysed relatively to the ribosomal protein  
192 RbL8 expression (Chevalier *et al.*, 2012), using LightCycler® 480 Software. Statistics on the tissue  
193 and development RT-qPCR data were calculated using the Kruskal-Wallis test, complemented by  
194 Dunn's *post-hoc* tests implemented in the R package PMCMR (Pohlert, 2014). The resulting p-  
195 values were adjusted using Holm's correction in order to take multiple comparisons into account.  
196 The Wilcoxon-Mann-Whitney test was used to assess the efficiency of the gene silencing in the  
197 RNAi experiment.

198

#### 199 2.4. Sequencing

200 The 26 IGFBP-rP1 sequences amplified by RT-PCR were treated with 2 µL of EXOAnP  
201 solution (0.05 µL of exonuclease I (1 u), 0.1 µL of AnP (0.5 u) and 1.85 µL of sterile water) at 37°C  
202 for 1 h. Enzymes deactivation was achieved at 80°C for 20 minutes. New PCR using the BigDye®  
203 Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) were then performed (3 µL of PCR  
204 product, 0.5 µL of BDT, 0.32 µL of primer, 3 µL of 5X buffer and sterile water to 15 µL). The  
205 products of the PCR were precipitated with a mix of 50 µL ethanol (100%), 2.2 µL NaOAc (3M,  
206 pH 5.2) and 10 µL water. Pellets were washed with 70% ethanol and solubilized in deionized  
207 formamide. Finally, DNAs were sequenced on both strands using Sanger method. The final 68

208 IGFBP-rP1 sequences were deposited in GenBank under accession numbers [pending submission].

209

## 210 2.5. RNA silencing

211 Av-AGH and Av-IGFBP-rP1 cDNAs were first generated by RT-PCR as described above,  
212 with specific primers (Table S2). Amplicons were purified using the QIAquick PCR Purification Kit  
213 (Qiagen) and then cloned using the pGEM®-T Easy Vector system, according to the supplier's  
214 instructions. Inserts were checked with Sanger sequencing as previously described. Single-stranded  
215 RNAs were generated in both directions, overnight, at 37°C the using MEGAscript T7 Kit  
216 (Ambion), treated with TURBO DNase (15 minutes at 37°C), precipitated with LiCl, washed with  
217 70% ethanol and dissolved in nuclease-free water, according to the manufacturer's instructions.  
218 Hybridisation of forward and reverse RNAs was performed first for 15 minutes at 70°C followed by  
219 10 minutes at room temperature. Double-stranded RNAs (dsRNAs) were quantified and diluted to  
220 1 µg/µL. Adult males were injected with 1 µL of dsRNA (Av-AGH or Av-IGFBP-rP1) or 1 µL of  
221 nuclease free water (vehicle group), to account for the trauma response, aging and environmental  
222 rearing conditions. These injections were performed using a Hamilton syringe through a hole drilled  
223 on the lateral side of the fifth segment of the pereion. Another control group was not injected at all.  
224 At one, four, 12 and 28 weeks post-injection, five animals of each group were frozen individually in  
225 liquid nitrogen for RNA extraction and another two were dissected to investigate the morphology of  
226 the androgenic glands (six per males) and the presence of female genital apertures.

227

## 228 2.6. Bioinformatics

229 The assembly of both newly generated and already published reads was achieved on our  
230 servers (Table S1). The raw reads were first filtered with Trimmomatic (leading and trailing quality  
231 of 5, minimum length of 36 bp, Illumina adapters removed) (Bolger *et al.*, 2014). The trimmed data  
232 were then assembled with IDBA-tran assembler (maximum k-mer size of 100 bp) (Peng *et al.*,  
233 2013). The IGFBP-rP1 sequences were searched locally with TBLASTX, using first Cq-IGFBP and

234 then Av-IGFBP-rP1 as queries.

235 The post-translational modifications were predicted using CBS prediction servers  
236 (SignalP 4.1 (Petersen *et al.*, 2011), NetPhos 3.1 (Blom *et al.*, 1999), ProP 1.0 (Duckert *et al.*,  
237 2004)). The conserved domains of the IGFBP-rP1 sequences were obtained using the Conserved  
238 Domain Search service (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) (Marchler-Bauer *et*  
239 *al.*, 2015). Coding sequences were retrieved with ORF Finder from the Sequence Manipulation  
240 Suite ([http://www.bioinformatics.org/sms2/orf\\_find.html](http://www.bioinformatics.org/sms2/orf_find.html)). Sequences logos were drawn using the  
241 WebLogo toolsite (<http://weblogo.berkeley.edu/logo.cgi>) (Crooks *et al.*, 2004).

242 Phylogeny reconstructions were carried out using the programs implemented in Seaview  
243 (Gouy *et al.*, 2010). Nucleotidic coding sequences were aligned using Muscle (Edgar, 2004). The  
244 final set of sites was determined with Gblocks (Castresana, 2000; Talavera and Castresana, 2007).  
245 The reconstructions themselves were performed using PhyML (maximum likelihood method  
246 (Guindon and Gascuel, 2003)), using GTR+I+G model of evolution as determined using  
247 jModelTest 2.1.9 (Darriba *et al.*, 2012; Guindon and Gascuel, 2003). Branch support was estimated  
248 with both the non parametric Shimodaira-Hasegawa-like approximate Likelihood-Ratio Test (SH-  
249 like aLRT) (Anisimova and Gascuel, 2006; Guindon *et al.*, 2010) and the bootstrap proportions  
250 (1000 repetitions) (Felsenstein, 1985). Branch supports were further checked with a bayesian  
251 method using BEAST 1.8.4 (Drummond *et al.*, 2012), using the same model of evolution, an  
252 uncorrelated relaxed clock (log normal) and a run of 50,000,000 generations (log sampling every  
253 1000 generations). Posterior probabilities (PP) were retrieved using TreeAnnotator.

254 Evidence for natural selection in our IGFBP-rP1 dataset was investigated using PAML  
255 (Yang, 1997, 2007). An overall dN/dS value was calculated with codeml (seqtype=1, model=0). A  
256 dN/dS value was calculated for each site with the tools available on the Datamonkey server (SLAC,  
257 FEL, REL (Kosakovsky Pond, 2005; Murrell *et al.*, 2012)) (<http://datamonkey.org>) (Pond *et al.*,  
258 2005; Pond and Frost, 2005).

259

## 260 **3. Results**

### 261 *3.1. Av-IGFBP-rP1 sequence and structure*

262 With a transcriptomic approach using publicly available reads, we identified in *A. vulgare* a  
263 unique transcript coding for the decapod homologue of IGFBP-rP1, named Av-IGFBP-rP1. Its  
264 sequence was confirmed using the Sanger method, revealing a coding sequence of 744 bp  
265 (247 amino acids). A signal peptide was predicted, with a cleavage site between positions 22 and 23.  
266 Two sites of phosphorylation (Ser145, Ser232) but no R/K cleavage site were predicted.

267 In keeping with descriptions in Decapoda, three conserved domains were predicted in Av-  
268 IGFBP-rP1. It included the IB domain itself, to which the ligand binds, followed by the Kazal  
269 domain (a serine protease inhibitor) and finally an Immunoglobulin I-set domain (Fig. 1).

270

### 271 *3.2. Expression analysis of Av-IGFBP-rP1*

272 The spatial expression of the Av-IGFBP-rP1 mRNA was investigated by RT-PCR (Fig. S1)  
273 and RT-qPCR (Fig. 2) in adult *A. vulgare*. Av-IGFBP-rP1 was expressed in all the individuals,  
274 regardless of their sex (male, female or male intersex) or their *Wolbachia*-infection status. In all  
275 cases, we found a broad expression of Av-IGFBP-rP1 in the analysed tissues. The RT-qPCR  
276 analyses in the uninfected lineage showed that this expression was especially strong in the nerve  
277 cord (NC), the muscles and fat (MF), the digestive tract (DT) and the ovaries (OV) (Fig. 2A). In  
278 contrast, this expression was especially weaker in the digestive *caeca* (CK). The same expression  
279 pattern was observed in the *Wolbachia*-infected lineage (Fig. 2B) and in the intersexes generated  
280 experimentally by injecting *Wolbachia* in uninfected males (Fig. 2A).

281 The expression of the Av-IGFBP-rP1 mRNA was also investigated in whole individuals at  
282 each developmental stage by RT-qPCR (Fig. 3). In the uninfected lineage, Av-IGFBP-rP1 gene  
283 expression was elevated after birth (week 0), decreased after the first and second weeks and  
284 remained stable until adulthood (stages 4-9) (Fig. 3A). A similar expression pattern was observed  
285 during the development in the *Wolbachia*-infected lineage (Fig. 3B). Overall, this gene was found



286 continuously expressed throughout development.

287

### 288 *3.3. Silencing of Av-IGFBP-rP1 and Av-AGH*

289 To confirm the implication of Av-IGFBP-rP1 in the ISP, its cross-talk with Av-AGH was  
290 investigated using a RNA silencing approach (Fig. 4). One week post-injection (wpi) of the Av-  
291 AGH dsRNA, the expression of Av-AGH was successfully inhibited by 98% compared to the  
292 vehicle group ( $p=0.001$ ). The silencing efficiency was still of 95% ( $p=0.001$ ), 89% ( $p=0.002$ ) and  
293 64% ( $p=0.032$ ) at four, 12 and 28 wpi, respectively (Fig. 4A). In the same way, the expression of  
294 Av-IGFBP-rP1 in the IGFBP-rP1 silencing experiment was reduced by 88% ( $p=0.001$ ), 86%  
295 ( $p=0.001$ ), 94% ( $p=0.002$ ) and 92% ( $p=0.008$ ) at one, four, 12 and 28 wpi, respectively (Fig. 4B).  
296 Whereas the inhibition of the Av-AGH gene did not impact the expression of the Av-IGFBP-rP1  
297 gene (Fig. 4C), the silencing of Av-IGFBP-rP1 gene induced an increase in the Av-AGH mRNA  
298 level, which significantly reached a two-fold increase at four ( $p=0.019$ ) and 12 wpi ( $p=0.002$ ), and  
299 up to a seven-fold increase at 28 wpi ( $p=0.016$ ) (Fig. 4D).

300 The impact of these two silencing experiments on the gonad phenotypes was also  
301 investigated (Fig. 5). No altered phenotype could be observed one week after the silencing of both  
302 genes. Four weeks after AGH dsRNA injection, half of the investigated androgenic glands appeared  
303 hypertrophied (bigger and more nodular) (5/10) (Fig. 5B). Four weeks after IGFBP-rP1 dsRNA  
304 injection, hypertrophied androgenic glands also began to appear (2/10) (Fig. 5E). At 12 and 28 wpi,  
305 we observed hypertrophy for all of the androgenic glands in the AGH silencing experiment (12/12  
306 and 12/12) (Figs. 5C and 5D), as well as the appearance of female genital apertures in half cases  
307 (Fig. 5H). In contrast, at 12 and 28 wpi in the IGFBP-rP1 silencing experiment, the androgenic  
308 glands seemed normal (hypertrophy: 0/12 and 0/9) in the dissected individuals (Figs. 5F and 5G),  
309 which did not display female genital apertures either (Fig. 5I).

310

### 311 *3.4. IGFBP-rP1 in Isopoda: sequences, structure and evolution*

312           Considering the constitutive expression of the IGFBP-rP1 gene in the different tissues and  
313 during the development of *A. vulgare*, as well as the consequence of the silencing experiment, we  
314 suspected an essential role of Av-IGFBP-rP1 in the ISP and thus investigated its global conservation  
315 in isopods. IGFBP-rP1 sequences were obtained using two complementary approaches: by RT-PCR  
316 with degenerated primers and by using both public and newly generated cDNA libraries. After the  
317 sequencing of the PCR fragments and assembly of the RNAseq data, a total of 68 IGFBP-rP1  
318 sequences was obtained from isopod species belonging to 18 different families: five consisting of  
319 aquatic species and 13 comprising terrestrial species (Table S1).

320           All of the sequences shared the canonical domains of this protein: the IB domain, the Kazal  
321 domain and an Immunoglobulin domain (Ig, Ig\_3) or Immunoglobulin I-set domain (Fig. 6). The  
322 open reading frame length varied between 720 bp (239 amino acids) for *Bragasellus peltatus* and  
323 *Proasellus ibericus* and 756 bp (251 amino acids) for *Armadillo officinalis*. About 68% (26/38) of  
324 the terrestrial isopods (Oniscidea) displayed an IGFBP-rP1 coding sequence of 744 bp (247 amino  
325 acids) (Fig. 6). Decapod IGFBP-rP1 sequences have a length within the same range, from 747 bp  
326 (248 amino acids) for Cq-IGFBP but up to 771 bp (256 amino acids) for *Sagmariasus verreauxi*. In  
327 isopods, 74 amino acids were found completely conserved in all the sequences (Fig. 6). They  
328 constituted ~30% of the total sequence and were especially represented in the Ig domain (47/101).  
329 Among them, ~25% were cysteines (18 residues), mainly distributed in the IB domain (10/60) and  
330 the Kazal domain (4/39) but rare in the Ig domain (2/101) or out of the conserved domains (2).  
331 Several conserved motifs are described as being implicated in ligand binding: (R/L)<sub>x</sub>L<sub>xx</sub>LL in  
332 humans (Imai *et al.*, 2000; Hong *et al.*, 2002), RxL<sub>xx</sub>L in decapods (Rosen *et al.*, 2013) and  
333 CGCC<sub>xx</sub>C in vertebrates (Hwa *et al.*, 1999; Daza *et al.*, 2011). We found in isopod IGFBP-rP1 the  
334 same CGCC<sub>xx</sub>C determinant as the one identified in vertebrates (Fig. 6, framed in green). In  
335 contrast, the homologue region of the decapod motif RxL<sub>xx</sub>L (Fig. 6, framed in blue) did not  
336 correspond to the same sequence. However, a close and overlapping region was found to be similar  
337 to the described motif (Fig. 6, framed in orange), even if not fully conserved: (I/V)<sub>x</sub>L<sub>xx</sub>L (or

338 (I/V)<sub>xx</sub>LxL in *Proasellus sp.*). In this conserved determinant, the second conserved leucine in  
339 Isopoda was found homologous to the first conserved leucine of Decapoda.

340 A phylogeny of all the IGFBP-rP1 sequences in isopods was reconstructed using the whole  
341 coding sequence (Fig. 7). The phylogenetic tree of the gene was globally congruent with the known  
342 phylogeny of isopod species. In particular, the IGFBP-rP1 of terrestrial isopods clustered together,  
343 which is consistent with the monophyly of Oniscidea. More extensively, the monophyly of every  
344 isopod family was respected with two exceptions. Indeed, our two representatives of Platyarthridae  
345 (Fig. 7, in red) as well as the four species of Philoscidae (Fig. 7, in purple) did not cluster together.  
346 The monophyly of species classified in Philoscidae is however deemed unlikely (Schmidt, 2008).  
347 Because of the modularity of the IGFBP superfamily members, we also constructed phylogenies of  
348 the different IGFBP-rP1 conserved domains separately, to check whether they evolved similarly or  
349 not. However, considering the very high conservation of these domains (e.g. half of the amino acids  
350 in the Ig domain strictly conserved in Isopoda), the resolution of these trees was rather poor (data  
351 not shown).

352 To confirm the apparent good conservation of the isopod IGFBP-rP1, we calculated the  
353 dN/dS ratios for the whole sequence set and then for each site of the coding sequence. The overall  
354 dN/dS ratio of the isopod IGFBP-rP1 alignment was between 0.12 (codeml, model 0) and 0.17  
355 (SLAC and REL), implying a strong purifying selection on this sequence. This was further  
356 confirmed by SLAC, FEL and IFEL methods that predicted 177 (73%), 191 (79%) and 173 (72%)  
357 negatively selected codons, respectively, 166 (69%) of these codons being found under purifying  
358 selection by these three methods.

359

#### 360 **4. Discussion**

361 In this study, we identified and described the first molecular partner of the AGH in Isopoda,  
362 namely the malacostracan order in which this unique sex hormone was initially identified and  
363 characterized (Hasegawa *et al.*, 1987; Martin *et al.*, 1990). In the model *A. vulgare*, we found Av-

364 IGFBP-rP1 expressed constitutively, without significant difference between males, females or  
365 intersex individuals. The lack of clear differential expression between males and females in isopods  
366 is consistent with the fact that *Wolbachia*-uninfected females are still responsive to the AGH, as  
367 shown by the grafts of androgenic glands or the AGH injections (De Lattin and Gross, 1953;  
368 Legrand, 1954; Katakura, 1959; Katakura and Hasegawa, 1983). Yet, the expression of IGFBP-rP1  
369 in females could be surprising considering its putative role in male differentiation and that they do  
370 not express the AGH. Actually, it is likely that IGFBP-rP1 ensures several functions simultaneously  
371 within the organism, including ligand-independent functions (Hwa *et al.*, 1999) and functions  
372 associated with additional ligands. Indeed, the AGH is not the only known member of the ILP  
373 family in Crustacea and thus, not the only candidate ligand for IGFBP-rP1. For instance, four ILPs  
374 had already been discovered in 2011 in the branchiopod crustacean *Daphnia pulex* (Colbourne *et al.*,  
375 2011; Dirksen *et al.*, 2011) and two others were described in the decapod *Sagmariasus verreauxi* in  
376 2015 (Sv-ILP1, a possible homologue of DILP7) (Chandler *et al.*, 2015) and in 2017 (Sv-ILP2, a  
377 possible homologue of DILP8) (Chandler *et al.*, 2017). To this date, the function of these ligands  
378 cannot be investigated in Isopoda as no homologue of ILP1 or ILP2 was described in any isopod  
379 species. Lastly, the exact interactions between the mobile IGFBP-rP1 and its target tissues are not  
380 described yet in crustaceans, so that we cannot exclude variations in its function according to the  
381 targets, such as feeding activity in the midgut (Huang *et al.*, 2016) or ovarian development (Huang  
382 *et al.*, 2015, 2017) for instance.

383         The analysis of Av-IGFBP-rP1 expression in the different tissues revealed that its spatial  
384 expression in adults was very broad, despite the variation in intensity. This is consistent with  
385 previous descriptions in decapod models in which IGFBP-rP1 is also expressed in all of the  
386 investigated tissues but hemocytes in *Scylla paramamosain* (Rosen *et al.*, 2013; Chandler *et al.*,  
387 2015; Huang *et al.*, 2015, 2016; Li *et al.*, 2015; Song *et al.*, 2018). More precisely, we found the  
388 highest Av-IGFBP-rP1 expression in the nerve cord, muscles and digestive tracts, and the lowest  
389 expression in the hemocytes, testis and most notably in the digestive *caeca*. A similar expression

390 level of IGFBP-rP1 is reported in the hepatopancreas (i.e. digestive *caeca*) of *Callinectes sapidus*  
391 and *Eriocheir sinensis* (Huang *et al.*, 2016; Song *et al.*, 2018), whereas the other decapod IGFBP-  
392 rP1s display a high gene expression in this tissue. Contrary to Av-IGFBP-rP1, all of them also show  
393 an elevated expression in the testis. It seems however difficult to conclude on functional differences  
394 between these homologues on the basis of their gene expression patterns only. Considering both its  
395 broad expression and its role as a binding protein, circulating freely in the hemolymph, it seems  
396 plausible that IGFBP-rP1 protein is ubiquitous in the organism despite local transcriptional  
397 variations. Besides, this broad spatial expression pattern is consistent with the fact that the AGH  
398 action is not restricted to the male gonads, as this hormone indeed acts on several secondary sex  
399 characters (copulatory organs (Suzuki and Yamasaki, 1991; Takewaki and Nakamura, 1944),  
400 brushes on the pereopods, behaviour (Barki *et al.*, 2006, 2003; Karplus *et al.*, 2003)) and is  
401 expressed, in some decapod species, by several organs besides the androgenic glands (ovaries,  
402 hepatopancreas, nerve cord) (S. Li *et al.*, 2012; Chung, 2014; Huang *et al.*, 2014; Li *et al.*, 2015).  
403 Likewise, the broad occurrence in the organism is in line with IGFBP-rP1 binding several ILPs with  
404 different spatial expression profiles (e.g. Sv-ILP1, Sv-ILP2, Sv-IAG; Chandler *et al.*, 2015; 2017).

405 We also investigated the differential gene expression of Av-IGFBP-rP1 during *A. vulgare*  
406 development. In crustaceans, a similar analysis was performed on *Macrobrachium nipponense* only,  
407 in which Mn-IAGBP transcription tends to the increase, particularly at the beginning of sexual  
408 differentiation (Li *et al.*, 2015). By contrast, we observed a significant decrease of Av-IGFBP-rP1  
409 expression after birth, followed by a relatively constant expression. Moreover, the Av-IGFBP-rP1  
410 expression patterns between males and females and between infected and uninfected lineages were  
411 similar. This suggests that Av-IGFBP-rP1 is required throughout the life of the animal but that other  
412 factors may account for the different developmental paths taken from stage four on by the males  
413 and the females or feminized genetic males. Despite the absence of differential gene expression  
414 patterns, there are other ways to modulate IGFBP-rP1 activity besides variations of transcription  
415 intensity. First, the amount of proteins could be regulated at the translation level, which would

416 require quantifying the IGFBP-rP1 protein. Then, the responsiveness of the different tissues, in  
417 particular the gonads, may also fluctuate: undifferentiated gonads may not interact in the same way  
418 with IGFBP-rP1 during development or according to their *Wolbachia*-infection status. Further  
419 studies should also investigate the affinity of isopod IGFBP-rP1 for its ligand in different contexts.  
420 IGFBP-rP1 pull-down combined with AGH antibodies may reveal affinity variations between sexes  
421 or between infected and uninfected lineages.

422 In that respect, a role of IGFBP-rP1 in the sex differentiation cannot be excluded,  
423 particularly since its implication in the AGH pathway is now well established in decapods  
424 (molecular interaction, modelling, silencing...). The isopod model also appeared particularly well-  
425 suited for the gene silencing approach, as only one injection of Av-AGH or Av-IGFBP-rP1 dsRNA  
426 could successfully inhibit the expression of the target genes for at least six months. This way, the  
427 implication of Av-IGFBP-rP1 in the AGH pathway was also confirmed in Isopoda with RNAi  
428 experiments. Indeed, both Av-AGH and Av-IGFBP-rP1 knock-down could induce the hypertrophy  
429 of the androgenic glands, which is also observed in naturally *Wolbachia*-induced intersexes.  
430 However, only the silencing of the Av-AGH induced the appearance of female genital apertures like  
431 in intersexes, leading to a more feminized phenotype. Moreover, the silencing of the Av-AGH did  
432 not affect the transcriptional level of Av-IGFBP-rP1, whereas the silencing of the latter induced the  
433 overexpression of the Av-AGH gene. Interestingly, the genetic interactions between Av-AGH and  
434 Av-IGFBP-rP1 are not consistent with the observations on *M. nipponense*, in which Mn-IAGBP  
435 silencing reduces the transcriptional level of Mn-IAG in the androgenic glands and conversely (Li  
436 *et al.*, 2015). To better understand the divergent results, it would be interesting to know whether the  
437 authors also observed altered phenotypes of the androgenic glands during the Mn-IAGBP silencing  
438 experiment.

439 The importance of IGFBP-rP1 in isopod biology was finally considered on the evolutionary  
440 scale. In the whole Isopoda order, each of the 68 studied species, both aquatic and terrestrial,  
441 displayed a single sequence of IGFBP-rP1. All of them featured the three same conserved domains

442 already described in decapods (Rosen *et al.*, 2013; Chandler *et al.*, 2015; Huang *et al.*, 2015, 2016;  
443 Li *et al.*, 2015; Song *et al.*, 2018). This description, associated with the monophyly of this gene in  
444 all Metazoa (Huang *et al.*, 2015), definitely proves that, despite the varying nomenclature, all  
445 homologue genes identified in crustaceans derived from only one ancestral sequence. Furthermore,  
446 the various sequences quite grouped according to their taxonomic origin in the phylogenies, both on  
447 the isopod (present study) or metazoan scales (Chandler *et al.*, 2015; Huang *et al.*, 2015, 2016).  
448 This is inconsistent with the observation of Li *et al.* (2015) regarding a clade gathering vertebrate  
449 and arachnid sequences, which can be explained by the absence of tree root in their analysis. Our  
450 study, in addition with the purifying selection brought out by the dN/dS analyses, strongly suggests  
451 that the function of the IGFBP-rP1 has been well conserved during malacostracan evolution. This is  
452 also supported by the conservation in the isopod sequences of most of the negatively charged amino  
453 acids in the N-terminal region of IGFBP-rP1 identified in Decapoda to be involved in electrostatic  
454 interaction with the ligands (Chandler *et al.*, 2017).

455         Studies on the evolution of IGFBP1-6 in vertebrates already revealed a complicated scenario  
456 although they are all composed of the same two conserved domains and form a clear monophyletic  
457 group (Daza *et al.*, 2011). By contrast, the evolutionary story of the IGFBP superfamily in  
458 invertebrates appears even trickier to decipher, all the more so because various proteins sharing  
459 partial similarity have now been identified in this superfamily. In Hexapoda for instance, the gene  
460 called IGFBP7 but also known as ImpL2 (Ecdysone-inducible gene L2) displays only the Ig domain,  
461 so that only this part of the sequence is homologue to the other members of the IGFBP-rP1 family  
462 (Bader *et al.*, 2013). This receptor is however able to bind ILPs (Bader *et al.*, 2013; Honegger *et al.*,  
463 2008). The Insulin-related peptide-Binding Proteins (IBPs) display only Ig domains as well, and are  
464 also able to bind several ILPs, including vertebrate ones (Gao *et al.*, 2012; Sloth Andersen, 2000).  
465 Rosen *et al.* (2013) reported ImpL2 and IBPs to be, together with Cq-IGFBP, three IGFBP  
466 homologues but their structural domains clearly differ, explaining why they were excluded in  
467 broader analyses such as in Huang *et al.* (2015). In the same way, Single Insulin Binding Domain

468 proteins (SIBDs), as their name implies, only display the IB domain, like neuroparsins (NP), which  
469 are also invertebrate peptides with only the IB domain (Badisco *et al.*, 2007). All these proteins  
470 (IGFBP, IGFBP-rP, IBP, ImpL2, NP, SIBD) are visibly related, yet distantly (Rodgers *et al.*, 2008).  
471 But considering the divergence between their sequences and given that none of the conserved  
472 domain is shared by all of them, establishing global phylogenies of this superfamily by classical  
473 means is impracticable, all the more so when exon shuffling events are likely to be involved  
474 (Rodgers *et al.*, 2008).

475         The high conservation of the IGFBP-rP1 sequences in malacostracans, as reflected by the  
476 same transcript structure and the close homology, suggests that Av-IGFBP-rP1 is a key element of  
477 the ISP, which plays the same role previously described with the decapod IGFBP-rP1, that is the  
478 binding of the AGH, among other ILPs. However, the different IGFBP-rP1 expression patterns  
479 during development and the different consequences of the IGFBP-rP1 silencing experiments also  
480 suggest that its exact function is not that well conserved between the decapod and isopod models.  
481 Further studies on the sexual differentiation in Isopoda will likely focus on a more direct evidence  
482 of the interaction between the IGFBP-rP1 and the AGH, as well as a more global integration of  
483 these two interacting molecules in the ISP. Indeed, several other promising binding or receptor  
484 proteins of ILPs have now been characterized in crustaceans. Firstly, SIBDs have been described in  
485 several decapod species, in which they were thought to be involved in the immune response  
486 (Castellanos *et al.*, 2008; Gai *et al.*, 2010) but likely ensure several functions (Chandler *et al.*, 2015).  
487 Lastly, new transmembrane receptors of the AGH were recently described in three decapod species:  
488 the Tyrosine Kinase Insulin-like Receptors (TK-IR), also called IAG receptors (IAGR) (Aizen *et al.*,  
489 2016; Sharabi *et al.*, 2016; Guo *et al.*, 2018). Two of these receptors have just been identified in the  
490 isopod species *P. beticus* and *A. vulgare* (Herran *et al.*, 2018), whereas the AGH remains the only  
491 ILP identified in this order so far.

492

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499

500 **Fig. 1.** Comparison of the conserved domains in the published decapod IGFBP-rP1s and the newly  
501 characterized isopod IGFBP-rP1, the sequence of *A. vulgare* being shown for illustration. The  
502 conserved domains were mapped using CDsearch (Marchler-Bauer *et al.*, 2015).

503

504 **Fig. 2.** Tissue expression of the Av-IGFBP-rP1 mRNA by RT-qPCR. The following tissues were  
505 dissected from *A. vulgare* adults of uninfected (A) and *Wolbachia*-infected lineages (B): HE:  
506 hemocytes, LE: legs, MF: muscle and fat, NC: nerve cord, BR: brain, SG: salivary glands, DT:  
507 digestive tract, CK: digestive *caeca*, OV: ovaries, SP: sperm cells, VD: *vasa deferens*, SV: seminal  
508 vesicle, UT: utricles, AG: androgenic glands.

509

510 **Fig. 3.** RT-qPCR expression profiles of the Av-IGFBP-rP1 mRNA during development in  
511 uninfected (A) and *Wolbachia*-infected lineages (B). *Larvae* were sampled after birth (0w), one  
512 (1w), two (2w) and three weeks (3w) after birth. Then, each step represents a developmental stage  
513 (4-8), characterized by a new moult, until adulthood (AM for adult males, AF for adult females).  
514 External sex characters start to appear at stage 5, allowing gender-specific sampling.

515

516 **Fig. 4.** RT-qPCR expression profiles of the Av-AGH mRNA in the Av-AGH silencing experiment  
517 (A), the Av-IGFBP-rP1 mRNA in the Av-IGFBP-rP1 silencing experiment (B), the Av-IGFBP-rP1  
518 mRNA in the Av-AGH silencing experiment (C) and the Av-AGH mRNA in the Av-IGFBP-rP1  
519 silencing experiment (D). Two kinds of controls were included: animals which were not injected

520 (NI, in green) and animals injected with water (in blue), which correspond to the vehicle group. The  
521 dsRNA-injected animals appear in red. Animal samplings were performed one, four, 12 and 28  
522 weeks post-injection (wpi).

523

524 **Fig. 5.** Phenotypes of the AG in the silencing experiment. Normal AG in the water-injected group  
525 (A). Hypertrophied AG in the AGH silencing group at four (B), 12 (C) and 28 (D) weeks post-  
526 injection of the dsRNA (wpi). Hypertrophied AG in the IGFBP-rP1 silencing group at four wpi (E),  
527 then non-hypertrophied AG at 12 (F) and 28 (G) wpi. Female genital aperture observed during AGH  
528 silencing, 12 wpi (H) but absent during IGFBP-rP1 silencing (I). The dashed lines show the plane of  
529 bilateral symmetry whereas the dashed circle shows the female genital aperture.

530

531 **Fig. 6.** Analyses of IGFBP-rP1 conservation in isopod crustaceans. An analysis of the amino acid  
532 conservation was carried out with Multiple Align Show on the alignment of all the isopod IGFBP-  
533 rP1 sequences (A). Fully conserved amino acids are highlighted in black, substitutions leading to a  
534 functional conservation of the amino acid are indicated in grey. The three conserved domains are  
535 framed in red. Sequence logos of the three conserved domains of the IGFBP-rP1 (IB, Kazal, Ig)  
536 were generated by WebLogo (B). Positively charged amino acids (KRH) are depicted in red,  
537 negatively charged amino acids (DE) in blue, polar amino acids (STNQ) in green, apolar amino  
538 acids (AVLIWFM) in purple and the others (YGPC) in black. The conserved ligand-binding motifs  
539 are framed in green (CGCCxxC) and orange ((I/V)xLxxL). The region homologous to the decapod  
540 motif is framed in blue.

541

542 **Fig. 7.** Isopod IGFBP-rP1 phylogeny of the whole cDNA. Sequences aligned with Muscle were  
543 trimmed with Gblocks (723 nucleic sites left). A maximum likelihood tree was constructed using  
544 PhyML, with GTR+I+G model of evolution and 1000 bootstrap repetitions. Branch supports were  
545 confirmed with SH-aLRT and bayesian approaches. Family names are indicated in black except for

546 families of aquatic species (in blue) and families in which the IGFBP-rP1 sequences did not cluster  
547 together (in red and purple).

548

549 **Fig. S1.** Tissue expression of the Av-IGFBP-rP1 mRNA by RT-PCR. The following tissues were  
550 dissected from *A. vulgare* adults of each sex (F: females, M: males, iM: male intersex) from  
551 uninfected (Wo-) and *Wolbachia*-infected (Wo+) lineages. AG: androgenic glands, BR: brain, CK:  
552 digestive *caeca*, DT: digestive tract, HE: hemocytes, MF: muscle and fat, NC: nerve cord, OV:  
553 ovaries, SV: seminal vesicle, UT: utricles, VD: *vasa deferens*.

554

555 **Table S1.** Species for which an IGFBP-rP1 sequence was obtained using RT-PCR or RNAseq.  
556 GenBank accession numbers are given for the cDNA libraries assembled using publicly available  
557 reads and for all IGFBP-rP1 sequences identified in this study.

558

559 **Table S2.** Primers used to amplify the IGFBP-rP1 sequences by RT-PCR, to amplify the Av-IGFBP-  
560 rP1 and RbL8 sequences by RT-qPCR and to amplify the Av-AGH and Av-IGFBP-rP1 sequences  
561 for gene silencing. The IGFBP-rP1 primers were designed to amplify only the cDNA and do not  
562 amplify genomic DNA.

563

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565

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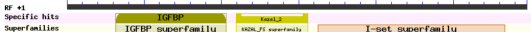
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Av-IGFBP-rP1



Cas-ILBP

KX267708.1



Cq-IGFBP

KC952011.1



Es-IGFBP7

KU724193.1



Mn-IAGBP

KJ831648.1



Sp-IGFBP-rP1

KR947299.1

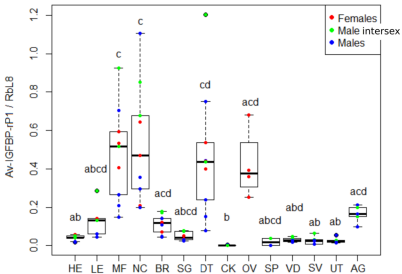


Sv-IGFBP

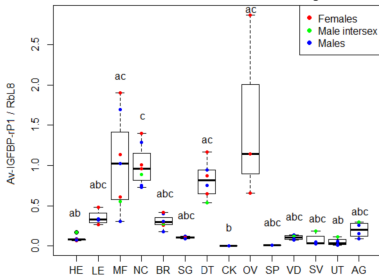
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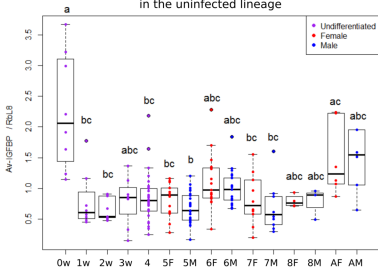
**A.** Relative expression of Av-IGFBP-rP1 in tissues of the uninfected lineage



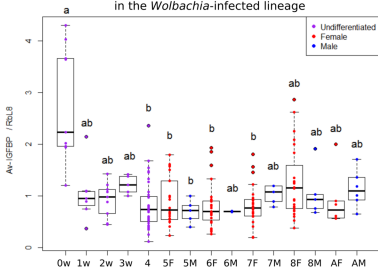
**B.** Relative expression of Av-IGFBP-rP1 in tissues of the *Wolbachia*-infected lineage



**A.** Relative expression of Av-IGFBP-rP1 during development, in the uninfected lineage



**B.** Relative expression of Av-IGFBP-rP1 during development, in the *Wolbachia*-infected lineage



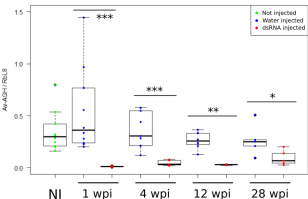
Embryos

Undifferentiated larvae

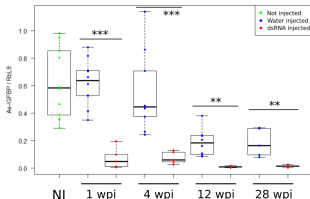
Differentiating juveniles

Mature adults

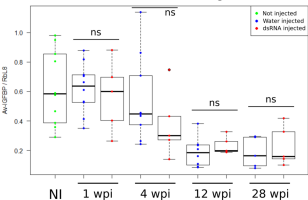
**A.** Relative expression of Av-AGH, in AGH silencing



**B.** Relative expression of Av-IGFBP-rP1, in IGFBP-rP1 silencing



**C.** Relative expression of Av-IGFBP-rP1, in AGH silencing



**D.** Relative expression of Av-AGH, in IGFBP-rP1 silencing

