Evaluation of the Baermann–Wetzel method for detecting lungworm larvae in wild ruminants from faecal samples

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Abstract

Lungworms can exert a negative impact on wild ruminant fitness, for this reason, the diagnosis of the associated diseases is an important prevention measure. The Baerman-Wetzel technique is the most usual method for the diagnosis of bronchopulmonary nematodes and is based on the active migration and movement of their first-stage larvae (L1). Pulmonary tissue samples are frequently used for the diagnosis of these parasites, but this kind of sample is not always available and easy to obtain. Faecal samples represent a more accessible choice for parasite monitoring. This work aimed to evaluate the agreement between the results obtained by the Baermann–Wetzel technique when samples of lung parenchyma or faeces from wild ruminants are used. A good level of agreement as well as a similar sensitivity between the two types of sample were observed, validating the use of faecal samples as a less invasive and cost-effective alternative for the monitoring of lungworm in wild ruminant populations.

Key words: Baermann–Wetzel technique; Bland-Altmann; Diagnostic; Lungworms; wild ruminant.

37 **1. Introduction**

38 Bronchopulmonary verminosis in wild ruminants is caused by nematodes that
39 inhabit bronchi, bronchioles and pulmonary alveoli. After ingestion, the third-
40 stage larvae (L3) migrate from the digestive tract to the lungs, where they molt to
41 the fourth stage (L4) in the bronchi and bronchioles and develop into adults
42 (Panuska, 2006). Embryonated eggs are coughed up, swallowed, and first-stage
43 larvae (L1) develop in the small intestine and are released to the environment via
44 faeces (Levine, 1985). It is well known that bronchopulmonary nematodes in wild,
45 farmed, and domestic ruminants are causal agents of respiratory disease with
46 potentially serious consequences for the health of the host (Carreno and Hoberg,
47 1999).

48 Lungworms are common parasites in wild ruminants (Marreros et al., 2012;
49 Panadero et al., 2001). Some *Protostrongylus* and *Elaphostrongylus* spp have
50 been mentioned among the most important causes of disease in wild ruminants
51 (Alasaad et al., 2009; Vicente et al., 2005). Although most infections are
52 subclinical, the co-infection with other microparasites or stress conditions can
53 lead to the development of respiratory disorders such as pneumonia (Jenkins,
54 2001) and other diseases (Mansfield et al., 1993). Lungworms can thus exert a
55 negative impact on the individual health and population fitness of wild ruminants,
56 inducing loss of weight, diminished offspring, abortions, neonatal deaths, and
57 increased mortality (Berrag and Urquhart, 1996; Gunn and Irvine, 2003; Lavín et
58 al., 1997).

59 The Baermann–Wetzel technique, based on the migration and movement of L1,
60 is the most widely used diagnostic tool for bronchopulmonary nematodes
61 (Eysker, 1997; Rode and Jørgensen, 1989). There are discrepancies in the

62 diagnostic performance of the Baermann–Wetzel technique using different
63 sample types (lung parenchyma vs faecal), with a higher sensitivity for the
64 pulmonary ones (Díez-Baños and Hidalgo-Argüello, 2006). However, faecal
65 samples offer several advantages with respect to the pulmonary tissue: i) they
66 are easily obtainable in a non-invasive and cost effective manner; ii) no animals
67 are removed, so their obtention does not impact small or otherwise vulnerable
68 populations; iii) it enables to repeatedly sample and monitor populations with
69 marked or confined individuals in fenced areas, which makes it possible to follow
70 the evolution of the infection or to study the phenology of these parasites,
71 improving the knowledge of the temporal and spatial dynamics of the lungworm
72 at population level (Cassini et al., 2015; Kafle et al., 2015). While fecal samples
73 are routinely used for diagnosing livestock, the diagnosis of wild ruminants relies
74 on the lung parenchyma of hunted or already dead individuals (Panadero et al.,
75 2001; Viña et al., 2013).

76 Proper sampling and determination of wildlife endoparasitic helminths can be an
77 outright challenge, although in the case of bronchopulmonary nematodes it is
78 feasible through the use of faecal samples (Kafle et al., 2015). Fecal samples
79 collected in the field have the potential to answer many ecological questions that
80 may otherwise remain out of reach (Putman, 1984). Lungworms also present the
81 advantage of being thermostable for cryopreservation as well as drought-tolerant
82 in faeces (Andermatt-Mettler et al., 1987; Rose, 1957), easing the field-work and
83 allowing for long-term sample conservation, which can be key to carry out
84 comparative or retrospective studies. The evaluation of samples that bring
85 together the greatest possible number of advantages to detect and identify
86 bronchopulmonary nematodes at population and individual levels in wild

87 ruminants is key to correct management, especially for endangered host species
88 and in wild-domestic interface areas, where there is a potential for parasite
89 spillover (Bienioschek et al., 1996; Kelly et al., 2009; Panuska, 2006; Sleeman et
90 al., 2019). For this reason, this study aimed to evaluate the agreement between
91 the results obtained from lungworm monitoring in faeces and lung tissue from
92 wild ruminants using the Baermann-Wetzel technique by comparing (1) the total
93 number of larvae recovered from each type of sample, (2) the sensitivity of the
94 technique according to the type of sample used and (3) the agreement between
95 both sample sources to address a possible interchangeability in their use.

96 2. Material and methods

97 2.1. Type of samples, collection and storage

98 A total of 500 samples (n = 250 pulmonary samples; n = 250 faecal samples) was
99 obtained from four different host species of free-ranging wild ruminants hunted at
100 the Sierras de Cazorla, Segura y Las Villas Natural Park (SCSV), Jaen, Andalusia
101 (SE Spain): mouflon (*Ovis aries musimon*), red deer (*Cervus elaphus*), fallow
102 deer (*Dama dama*) and Iberian ibex (*Capra pyrenaica*). The respiratory tract,
103 including lungs and trachea, and rectal faecal samples were recovered from each
104 animal during the field necropsy. Faecal and pulmonary samples were kept in
105 pairs but in separate storage bags for one-to-one comparison and stored at -20ºC
106 until diagnostic analyses were performed.

107 2.2. Identification of parasites

108 The Baermann–Wetzel method was used to extract bronchopulmonary L1 as
109 described by Forrester and Lankester (1997). To process the respiratory system
110 of each animal, after opening the tracheobronchial tree, 25 g of trachea, bronchi,

111 and bronchioles were cut in pieces of 0.5 cm; subsequently, 0.5 cm diameter
112 pieces were taken from different parts of the parenchyma of both lungs, evenly
113 distributed, until a total of 25 g was collected. Each of these pooled samples was
114 immersed into a separate beaker filled with tap water. Likewise, 25 g of faeces
115 were processed as mentioned above. The samples were kept for 12 hours in the
116 Baermann apparatus, after which 10 ml of the fluid from the bottom of the
117 migration system were collected, centrifuged for 5 minutes (800 *g*), and the
118 sediment was saved. Larvae were counted under the microscope with a Favatti
119 chamber and then deposited on slides with the addition of a drop of lugol solution
120 to fix the L1; this semi-permanent preparation allowed the morphological
121 identification of the larvae to genus according to Pyziel, 2014 and van Wyk et
122 al., 2004. Up to 100 individual larvae per sample were determined. In the case of
123 samples with a lower number of lungworms, all specimens were identified.
124 Results were expressed as the number of L1 per gram of examined sample
125 (LPG).

126 **2.3. Statistical analysis**

127 The relative performance of the Baermann–Wetzel technique applied to two
128 different sample categories was assessed with several statistical indicators.

129 First, the sensitivity of the two approaches was calculated as the true positive
130 rate of positive samples (capacity of the test to correctly classify animals with
131 lungworm infection). Specificity was assumed to be 100% for both diagnostic
132 approaches, as the method allows a perfect identification of the parasites. In
133 other words, the percentage of false positive results is equal to zero.

134 Afterwards, to determine the agreement between LPG results obtained from lung
135 tissues and faeces, a comparison of the average differences of LPG was
136 performed using the Bland-Altman test (Bland and Altman, 1986). The method
137 considers the two sample types to be in agreement if their results fall within the
138 so-called Limit of Agreement (LoA) interval. This interval was calculated using
139 the mean difference ± 1.96 *SD* of the LPG obtained using both samples. LoA was
140 calculated for all the larvae together and then split by parasite genus.

141 Finally, correlation analysis was implemented as a third indicator of agreement
142 between samples. High correlation values between the results obtained on
143 pulmonary tissue and faecal samples was considered an indicator of a good
144 match between the two sampling approaches.

145 All analyses were carried out using RStudio software version 1.2.5033.

146 **3. Results**

147 **3.1. Overall bronchopulmonary larval frequency and intensity**

148 From the overall analyzed samples, 39.3% (n = 98) were positive when using
149 pulmonary tissue and 38.0% (n = 95) by faeces. Nonetheless, 45.2% (n = 113)
150 of the analyzed animals turned out positive with at least one method. These
151 numbers suggest a similar sensitivity for both methods (86.7% for pulmonary
152 tissue and 84.1% for faeces). On average, the parasite load was 61.4 ± 306.1
153 LPG, with different values according to the sample used: 47.3 ± 256.8 LPG on
154 pulmonary tissue, and 14.3 ± 66.2 on faeces. The LPG values obtained on the
155 two type of sample are represented in Figure 1–A.

156 **3.2. Evaluation of the agreement for total LPG**

157 The Bland-Altman test was used to evaluate the agreement between both
158 samples using the Baermann–Wetzel technique. As represented in Figure 1-B,
159 samples ranging from of 0 to 500 mean ± 1.96 *SD* LPG do not exceed the LoA,
160 showing that the two samples are in agreement and may be used
161 interchangeably. Nonetheless, for samples exceeding 500 LPG (n = 3), the two
162 methods were not in agreement, and the real LPG value was under or
163 overestimated. Interestingly, samples for which there is a lack of agreement are
164 the ones with very high parasite intensity (above 500 LPG). Finally, the overall
165 correlation between the values obtained using both sample types was quite high
166 and significant (R= 0.54; p <0.001 - Figure 1-C).

167 **3.3. Evaluation of the agreement for each genera LPG**

168 From the positive samples (n = 113), seven different bronchopulmonary
169 nematode genera were identified: *Muellerius* spp. (61/113; 53.9 %), *Cystocaulus*
170 spp. (54/113; 46.9 %), *Neostrongylus* spp. (39/113; 34.5 %), *Elaphostrongylus*
171 *cervi* (34/113; 30.0 %), *Protostrongylus* spp. (22/113; 19.4 %), *Varestrongylus*
172 spp (14/113; 12.3 %) and *Dyctiocaulus* spp. (11/113; 9.7%). As presented in
173 Figure 2, we performed the Bland-Altman agreement test for the one-to-one
174 comparison of each parasite genus following the same principle described above.
175 Considering the LPG values obtained in each of the four nematode genera
176 globally (*Dyctiocaulus* spp., *Protostrongylus* spp., *Neostrongylus* spp. and
177 *Muellerius* spp.) and in *E. cervi,* only one sample exceeded the LoA LPG. This
178 could be considered as an outlier present in this study (Fig. 2 A-E). The Blant-
179 Altman test showed a perfect agreement for *Dyctiocaulus* spp., *E. cervi* and
180 *Protostrongylus* spp. in the range from 0 to 40 LPG (LoA not exceeded) (Fig. 2
181 A-C). *Neostrongylus* spp. and *Muellerius* spp. showed agreement up to 200 LPG

182 (Fig. 2 D-E). Finally, all LPG values from both sample types positive to
183 *Varestrongylus* spp. and *Cystocaulus* spp. did not exceed the LoA (Fig. 2 F-G).
184 In general, for most of the genera there was a significant correlation between the
185 LPG values detected with the two types of sample, with an R value ranging
186 between 0.32 (*Protostrongylus* spp.) and 0.96 (*Dyctiocaulus* spp.) (Fig.3 A, C –
187 F and G) . No significant correlation was detected for *E. cervi* and *Varestrongylus*
188 spp. (Fig. 3 B and F).

189 **4. Discussion**

190 This study presents the evaluation and performance comparison of the
191 Baermann–Wetzel technique applied to two types of sample (pulmonary tissue
192 and faeces). Bronchopulmonary nematodes have been widely reported in wild
193 ruminants (Alasaad et al., 2009; Meana et al., 1996; Panadero et al., 2001), and
194 many studies are based on results obtained using the Baermann–Wetzel
195 technique on lung tissue samples (Panadero et al., 2001; Pyziel, 2014). In
196 contrast, the use of faecal samples for the diagnosis of lungworm infection in
197 livestock is usually carried out using faecal samples (Díez-Baños et al., 1994;
198 Viña et al., 2013). Our results allow us to evaluate the accuracy and agreement
199 of the results obtained with the two types of sample. Since both kinds of sample
200 come from the same animal, we can confidently apply correction factors to the
201 estimation of prevalence of lungworm and LPG values obtained from different
202 biological samples, which will undoubtedly serve as a reference for future studies
203 to be conducted only with the analysis of wild ruminant faeces. Our results are
204 helpful to understand when and under which circumstances the Baermann–
205 Wetzel technique can be applied in non-invasive sampling schemes to evaluate
206 the status and dynamics of lungworms in free roaming ruminant populations.

207 The Baermann–Wetzel method is considered the gold standard for the detection
208 of bronchopulmonary infections (MAFF, 1986), as described by different authors
209 who demonstrated its high sensitivity in detecting L1 (Eysker, 1997; Viña et al.,
210 2013). Sensitivity of the diagnostic test seems to reach up to 90% (Traversa et
211 al., 2008; Willard et al., 1988) and this finding has been confirmed by our results.
212 In fact, using both kinds of sample, the sensitivity is close to 90%. However, in
213 terms of LPG evaluation, the Baermann–Wetzel technique applied to faecal
214 samples always detected a lower number of L1 in comparison with lung tissue
215 samples. The variability between faeces and bronchopulmonary tissue might be
216 due to extrinsic factors (i.e.: period of sampling) as described for the chamois
217 (*Rupicapra rupicapra*) by Diez-Baños et al. (1990), or simply because of the lower
218 concentration of L1 in the faeces than in the pulmonary system due to the location
219 of the fertile adult nematodes.
220 Despite the different LPG detected, our results show a significant agreement
221 between faecal and pulmonary samples, as highlighted by the Bland-Altmann test
222 (Bland and Altman, 1986). This technique is considered the most appropriate
223 approach to evaluate the agreement between diagnostic techniques, instead of
224 the more widely used correlation analysis. The latter can be misleading, and in
225 our work have been used only as additional agreement indicators. Many
226 published methodological studies about diagnostic techniques for
227 bronchopulmonary nematodes support their conclusions only on the lack of
228 correlation between L1 found in faeces and pulmonary tissue as a way of
229 comparison (Díez-Baños and Hidalgo-Argüello, 2006), underestimating the
230 importance of faecal material as a sensitive and non-invasive monitoring
231 approach. Thus, we aimed for the first time to measure the agreement using a

232 different and more accurate approach, to better investigate and evaluate the
233 importance and potential use of faecal samples in the study of lungworm
234 dynamics in wild ruminant populations, endangered host species and zoo
235 animals (Cassini et al., 2015; Nocture et al., 1998).
236 In general, our study highlighted a consistent agreement in the diagnostic results
237 obtained with the two kind of samples. In particular, it is noteworthy the consistent
238 agreement highlighted for two lungworm genera, *Neostrongylus* spp. and
239 *Muellerius* spp., suggesting that for these two genera in particular it might be
240 possible to use both pulmonary or faecal samples for a proper sanitary
241 surveillance. These two nematode genera had the highest LPG values compared
242 to the other genera of lungworm isolated. Similar results have already been
243 described for the chamois by Diez-Baños et al. (1990), who also compared
244 pulmonary versus faecal samples and described an association of the above-
245 mentioned nematode genera thus, indicating a possible relation between
246 animals presenting co-infection and high LPG. Hence, naturally parasitism
247 infection occurring at high prevalence and LPG values seems to show an
248 important agreement between results from both kind of samples.
249 Our results show that the sensitivity of the Baermann–Wetzel technique using
250 faeces is comparable to that obtained when using pulmonary tissues. That is, our
251 study confirms that epidemiological or diagnostic studies of wild ruminant
252 lungworms can be perfectly performed with faeces, maintaining the same level of
253 accuracy as obtained using lung tissue, but with the advantage that faeces are a
254 non-invasive and easy to obtain sample. This should be taken into account by
255 field researchers in understanding the value of faecal collection for the
256 coprological measurement of L1 when necropsies are not possible nor

convenient (Marreros et al., 2012). Our findings also revealed that for highly infected animals the agreement between the two sampling approach is progressively lost. However, considering that in our study the lack of agreement has been observed in less the 1% of the examined animals, the problem can be considered negligible and not influencing the results of large-scale monitoring.

A correct and precise diagnosis of lungworm genera is the key for an accurate sanitary surveillance, long-term monitoring, treatment of affected animals, and as a basis for population management. Our work highlights the agreement of pulmonary versus faecal samples for one of the most commonly used diagnostic methods for lungworm infection, indicating that both types of samples can be used without losing sensitivity and accuracy. These results support the use and value of faeces as non-invasive and cost-effective sampling technique for long term studies as well as for the preservation and conservation of threatened wild ruminant populations.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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393 from a human shelter. J. Am. Vet. Med. Assoc. 192, 913–916.

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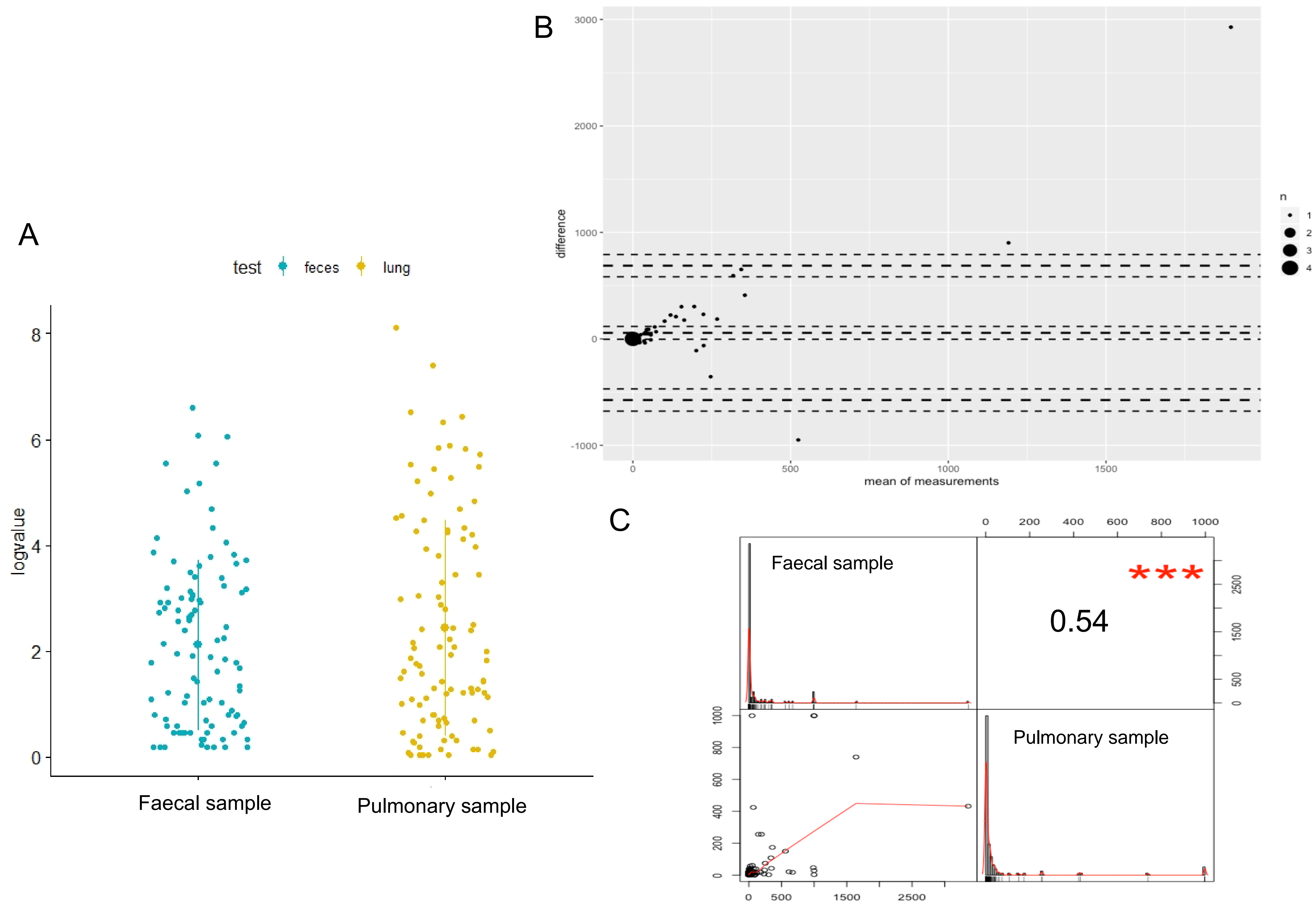


Fig.1: Graphical representation from the overall bronchopulmonary larval frequency (A) and the Bland-Altman results for the evaluation of the agreement between pulmonary and faecal samples using the Baermann–Wetzel technique (B). Additionally, correlation between the values of both sample types was calculated showing significantly high results (R= 0.54; *p* <0.001) (C).

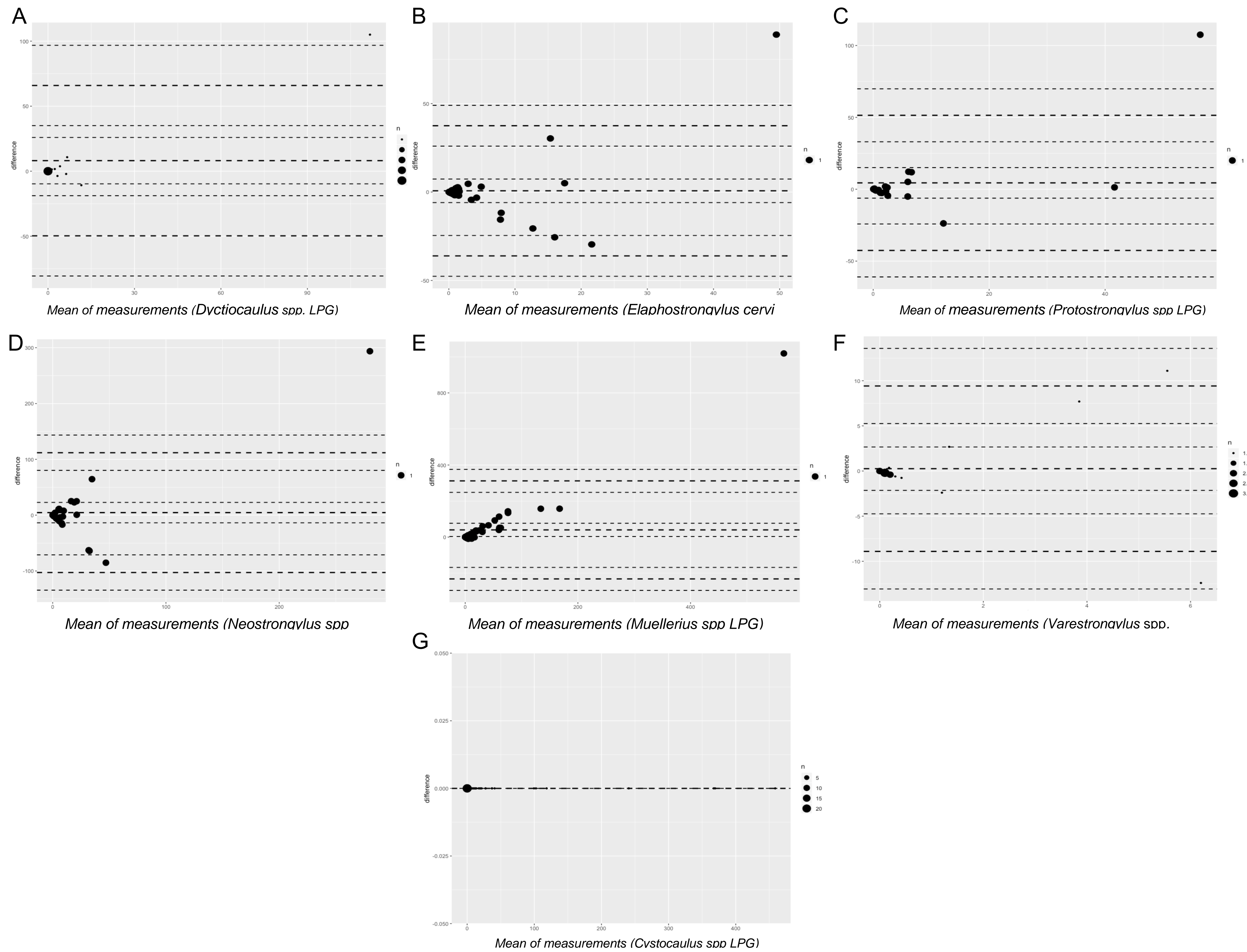
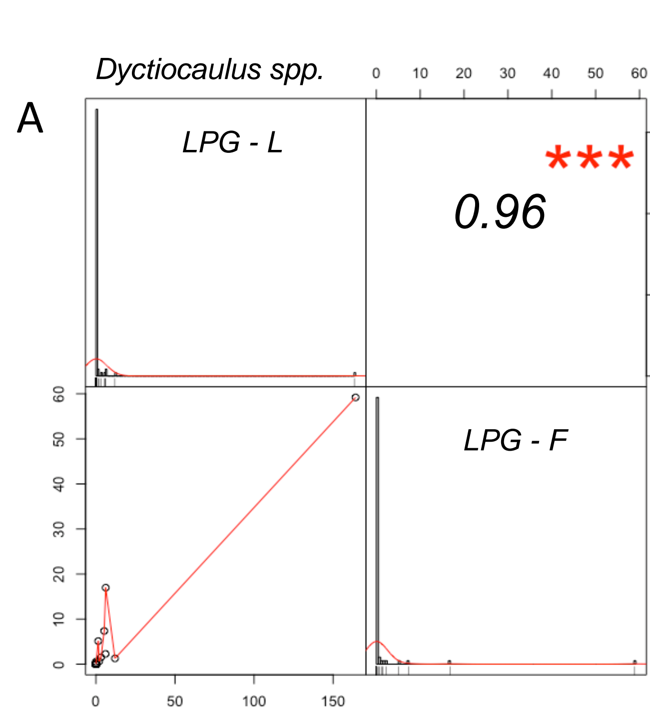
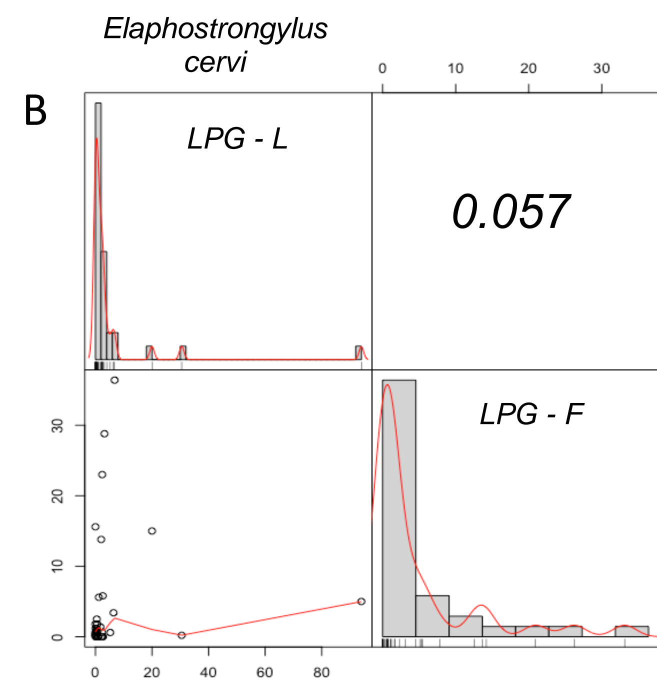
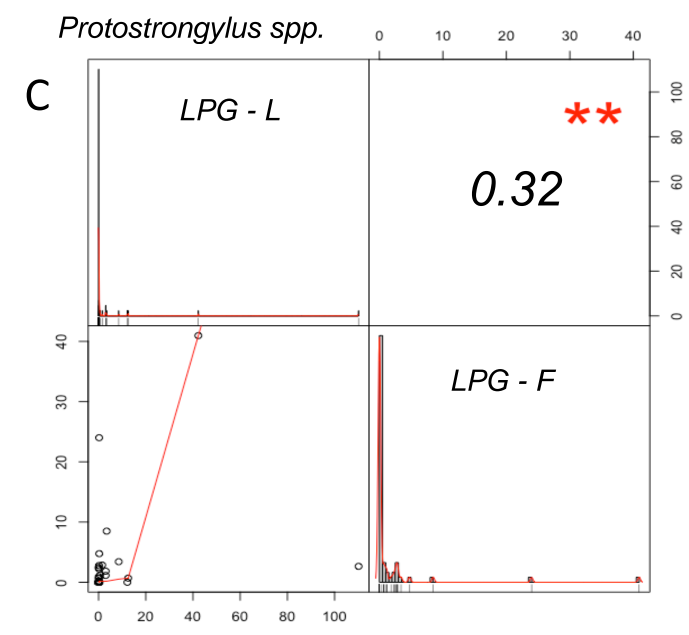
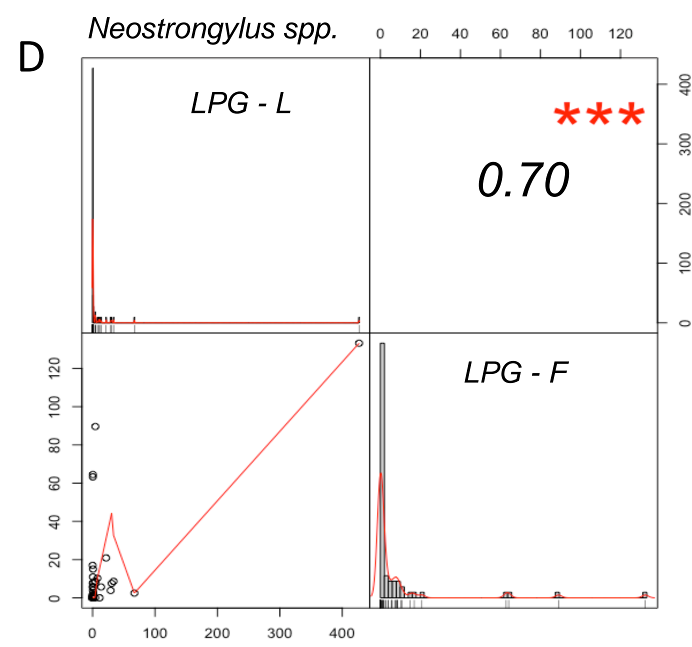


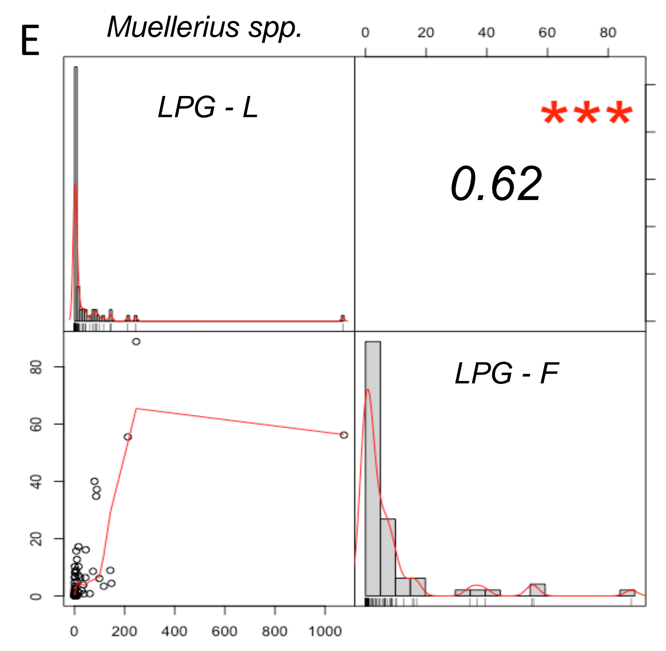
Fig.2: Bland-Altman representation of the agreement test for one-to-one comparison of each parasite genus between pulmonary and faecal samples using the Baermann–Wetzel technique (A-G). LPG: Larave per gram.

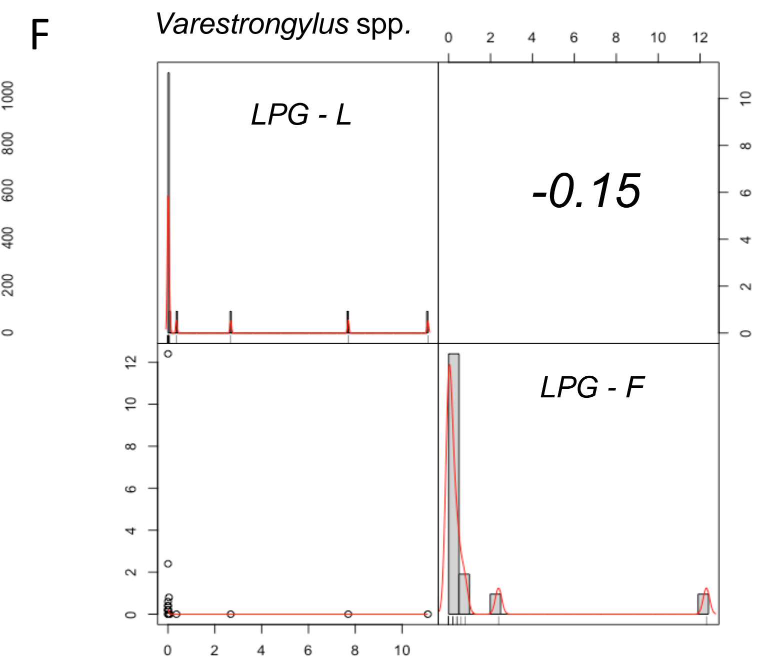

A
Dyctiocaulus spp.
LPG - L
***
0.96
LPG - F


B
Elaphostrongylus cervi
LPG - L
0.057
LPG - F


C
Protostrongylus spp.
LPG - L
**
0.32
LPG - F


D
Neostrongylus spp.
LPG - L
***
0.70
LPG - F


E
Muellerius spp.
LPG - L
***
0.62
LPG - F


F
Varestrongylus spp.
LPG - L
-0.15
LPG - F

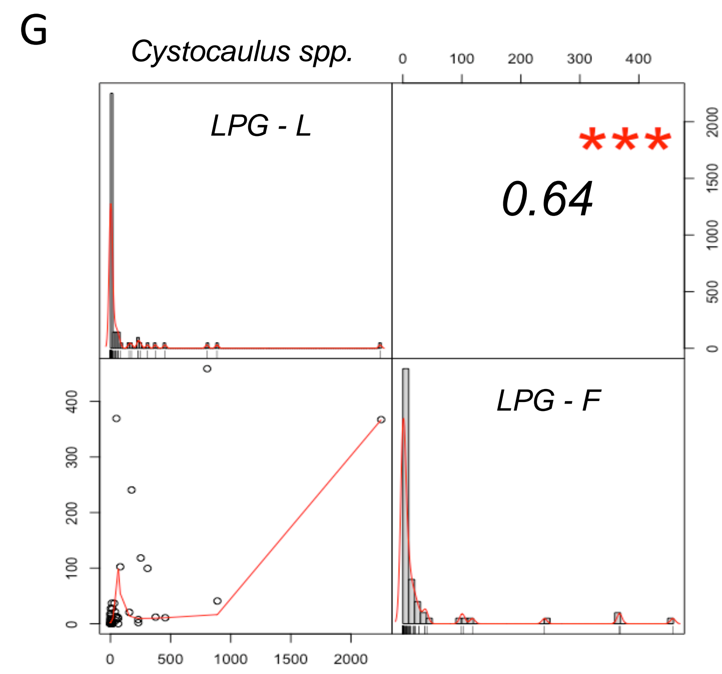

G
Cystocaulus spp.
LPG - L
***
0.64
LPG - F

Fig. 3: Graphical correlations of all identified genus and their significant reults between the LPG values detected with the two types of sample (A - G). LPG: Larave per gram; LPG - L: : Larave per gram in lung; LPG - F: : Larave per gram in faeces.