Title

Liver function markers and haematological dynamics during acute and chronic phases of experimental *Fasciola hepatica* infection in cattle treated with triclabendazole

Authors

Monique Costa[1], Anderson Saravia[2], Diego Ubios[3], Pablo Lores[1], Valeria da Costa[1], María Florencia Festari[1], Mercedes Landeira[1], Santiago A. Rodríguez-Zraquia[1], Georgget Banchero[3] and Teresa Freire[1]

Highlights

- *Fasciola hepatica* is a liver fluke that causes fasciolosis, which is usually treated with triclabendazole (TCZ)
- In this work we exhaustively analysed *F. hepatica* acute and chronic phases of infection in bovines.
- Increased levels of serum transaminase activity levels were detected in early stages of infection.
- Circulating eosinophil count and platelecrit were correlated with fluke number in livers from infected bovines.
- TCZ-treatment partially reduced parasite burden and liver damage.

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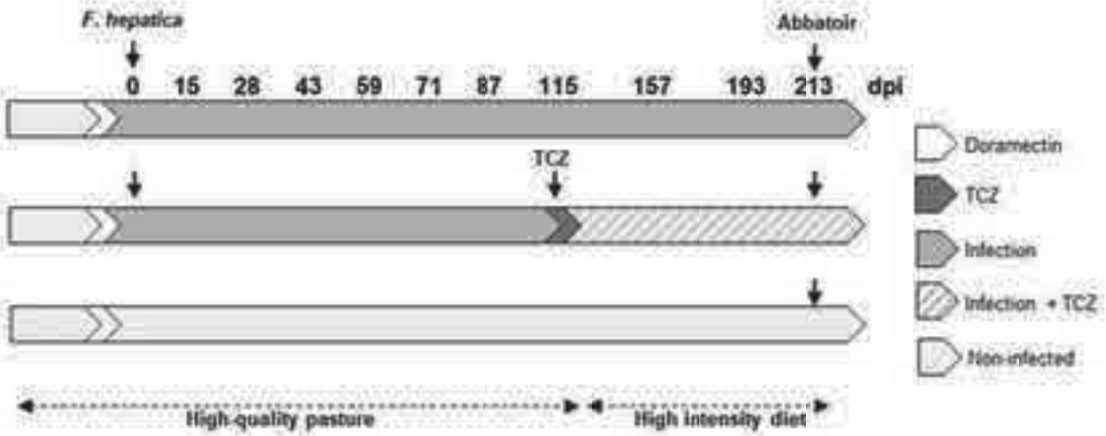
n=12/group



- Increased levels of serum transaminase activity levels were detected in early stages of infection
- Coprological method was not efficient to early diagnose bovine fasciolosis
- Circulating eosinophil count and platelecrit were correlated with fluke number in livers from infected bovines
- TCZ-treatment partially reduced parasite burden and liver damage, but did not avoid liver condemnation

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Abbreviations: TCZ: Triclabendazole; dpi: days post-infection; wpi: weeks post-infection

Abstract

Fasciola hepatica, a worldwide-distributed liver fluke, is one of the causative agents of fasciolosis, a zoonotic disease that affects livestock and humans. In livestock, fasciolosis causes huge economic losses worldwide, reducing animal fertility, milk production, weight gain and condemnation of livers. In spite of the availability of drugs, such as triclabendazole (TCZ), for the treatment of fasciolosis, they do not necessarily prevent liver damage or parasite reinfection and can eventually increase parasite resistance. The aim of this research was to relate the hepatic function, haematological parameters, leukocyte counts in circulation and parasite egg shedding during *F. hepatica* acute and chronic phases of infection in cattle as well as to determine how these parameters change with TCZ-treatment of chronically infected cattle. Our results show that increased levels of serum aspartate aminotransferase (AST) and gamma glutamyltransferase (GGT) were detected in early stages of the experimental infection. Moreover, high circulating eosinophil count and plateletcrit levels were correlated with fluke number in livers from infected cattle. On the other hand, although TCZ-treatment in the chronic phase of infection reduced parasite burden and damage in the liver, it was not able to completely avoid them. In conclusion, our work sheds light into the physiopathological mechanisms induced during fluke infection in cattle, revealing the complexity of the host response to the infection, together with the effects of TCZ-treatment in chronically infected animals.

Keywords

Fasciola hepatica, fasciolosis, liver, fluke, triclabendazole

1. Introduction

Fasciola hepatica, a worldwide-distributed liver fluke, is one of the causative agents of fasciolosis, a zoonotic disease that affects livestock and humans (1). Indeed, the World Health Organization estimates that around 17 million people are infected in more than 70 countries worldwide (2, 3). Furthermore, fasciolosis in livestock causes huge economical losses of approximately 3 billion dollars per year (1, 4, 5) due to reduced fertility and milk production, prevalence of secondary infections, reduced weight gain and condemnation of livers caused by chronic infections (6-8). In cattle, *F. hepatica* causes liver damage due to immature fluke migration and obstruction of the bile ducts by adult parasites (1, 5, 9). A recent review on fasciolosis epidemiology has reported its prevalence in ruminant species, with up to 91% in cattle some regions of Africa and South America (10). In Uruguay, the overall prevalence of bovine fasciolosis in abattoirs was recently reported to be about 34%, while in some territories of the country it increases up to 55%, implicating considerable economic losses considering only liver confiscation (11). Furthermore, this report also concluded that *F. hepatica* infection in Uruguayan cattle is associated with poorer carcass quality parameters and lower weights at slaughter, and the effect on weight differs across age ranges (11). The life cycle of *F. hepatica* is complex as the parasite goes through multiple stages before reaching its adult form and includes an intermediate host, a mud snail of the *Lymnaea* genus, and a definitive host, for instance livestock or humans (3, 5, 12). After ingestion of metacercariae, the infective form of *F. hepatica*, by the mammalian host, excystation occurs and the newly excysted juvenile flukes penetrate the host's intestine wall and reach the liver between 4-6 days. Juvenile flukes then burrow through the liver parenchyma for up to 6 weeks and damage considerable amount of tissue (5). Eventually, the flukes reach the bile ducts, where they become sexually mature and start to shed eggs from 12 weeks post-infection (wpi) (3), although these characteristics can change depending on the mammalian host taking up to 30 weeks for the parasite to complete

its life cycle (12, 13). Acute fasciolosis is generally characterized by the presence of immature flukes in the liver that destroy hepatic parenchyma and cause haemorrhage, extensive liver damage with fibrinous deposits on the capsule (13). Then flukes enter the bile ducts. Chronic phase of fasciolosis occurs when flukes, once inside the bile ducts, extensively ingest blood, damage the mucosa and cause cirrhosis, anaemia and hypoproteinaemia (13, 14). The damaged bile ducts become enlarged, or even cystic, and have thickened, fibrosed walls and, in cattle, they are usually calcified (13, 14). Chronic infection in cattle is usually analysed after 12 and up to 14 wpi (15, 16).

Triclabendazole (TCZ), a benzimidazole derivative, is usually used for the treatment of fasciolosis. However, this drug does not necessarily prevent liver damage induced by the parasite or parasite reinfection (17). Furthermore, although several studies have assessed the efficacy of TCZ against *F. hepatica*, it was determined by assessing post-treatment reduction in the faecal egg count (18-20), a parameter that is not necessarily correlated with the liver fluke burden (21, 22). Indeed, the detection of parasite egg shedding in faeces does not reflect parasite infection, since parasite eggs cannot be detected during the long prepatent period of 11-12 weeks post-infection and their shedding is discontinuous (21). Interestingly, a coproantigen ELISA test was demonstrated to be more sensitive than faecal egg count in experimental-infected cattle (20). Also, this study analysed the TCZ treatment on experimentally-infected cattle although no correlation with liver damage was included (20). Furthermore, while the detection of specific antibodies is more sensitive and sooner than coproantigen ELISA test, they persist after treatment with TCZ, while both coproantigen ELISA and faecal egg counts returned to negative status (20). In fact, the antibody response to infection is frequently associated with exposure to the infection and may not be useful for the diagnosis of natural *F.*

95 *hepatica* active infection (23, 24). Last, reports of resistance to TCZ are increasing (25),
96 evidencing one of the major drawbacks nowadays in the treatment of this disease.
97
98 The characterization of the pathological events induced by *F. hepatica* infections turns out to
99 be difficult when working in naturally infected cattle, because animals are permanently
100 challenged with parasite ingestion and the effects of the infection are deduced from the clinical
101 signs of the animals or the liver damage at slaughter. Different works have been carried out to
102 characterize damage caused by the parasite in the livers both in naturally and experimentally
103 infected cattle (16, 20, 23, 24, 26-31), although correlations with hepatic function and TCZ-
104 treatment for long periods after infection are scarce. The present work relates hepatic
105 dysfunction, together with haematological and circulating leukocyte parameters with liver
106 damage caused by *F. hepatica* in cattle through both the acute and chronic phases of the
107 infection during 30 wpi and to determine how these parameters change with TCZ-treatment of
108 chronically infected cattle.

2. Materials and Methods

2.1. Parasite experimental infection

Animal handling and experiments were carried out in accordance with strict guidelines and regulations from the National Committee on Animal Research (Comisión Nacional de Experimentación Animal, CNEA, http://www.cnea.gub.uy/, National Law 18.611, Uruguay), according with the ARRIVE guidelines and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All procedures involving animals were approved by INIA's Committee on Animal Research (CNEA Protocol Number: 0009/11). Eighteen- to twenty-four-month-old male Aberdeen Angus steers (mean body weight 421.88 ± 17,7 kg) were used in this experiment. Animals were negative to *F. hepatica* exposure determined by the sedimentation technique (32) in three previous consecutive tests (every 4-5 weeks) during 4 months before experimental infections. The animals were drenched on arrival with Dectomax® 1% (Doramectin 1g/100 ml) with the recommended dose (1 ml/50 Kg body weight) and remained free of gastrointestinal parasites (as determined by the modified McMaster technique (33) until *F. hepatica* experimental infection. Steers were kept outdoors and fed with high-quality pasture and water *ad libitum* at the experimental station of INIA La Estanzuela, Colonia, Uruguay. Animals were divided into three groups of 12 animals according to matched age and weight: i) infected, ii) infected and TCZ-treated, and iii) control groups (Figure 1). Experimental infections (n=24) were carried out with 500 TCZ-sensitive metacercariae (Ridgeway Laboratories, England) per animal, spread in saline solution, inserted into gelatine capsules (Torpac®) and delivered orally using a dosing gun. As control, non-infected steers (n=12) were maintained under the same conditions of infected animals during the experiment in a separate space. After 115 days post-infection (dpi), the second group of animals was treated with TCZ (12 mg/kg, Fasimec®, Novartis)

according to the recommendations of the drug supplier laboratory. One week after, animals were transported and fed with high-energy diets at the intensive animal farming facility (feedlot) of Marfrig Group in Fray Bentos, Río Negro, Uruguay. Establishments where animals were kept were free of snails. At day 213 post-infection animals were transported to an abattoir (Marfrig S. A. Tacuarembó) and sample collection, liver examination and fluke recovery were carried out (Figure 1).

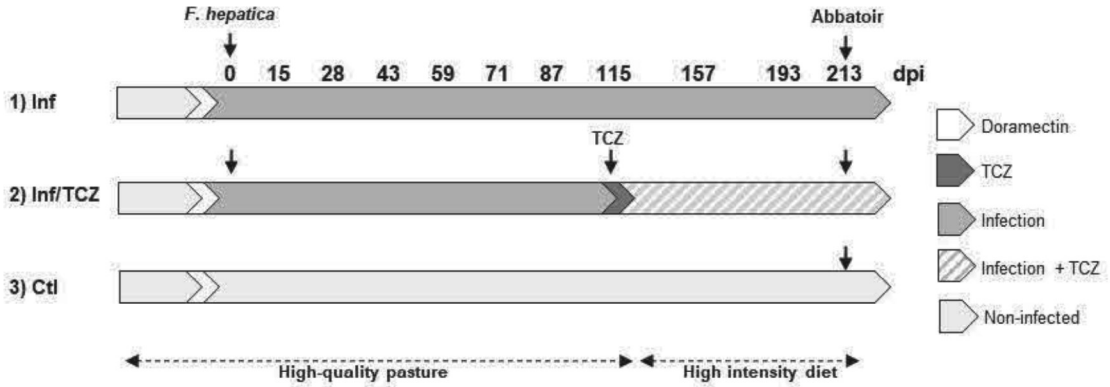

Figure 1. *F. hepatica* experimental infection timeline. Groups of 12 Aberdeen Angus steers were used per group. Animals from all groups were drenched with Doramectin to treat gastrointestinal parasite infection. Group 1 and 2 were infected with 500 *F. hepatica* metacercariae, while group 3 (control) remained non-infected. Animals were fed with high-quality pasture until day 122. At day 115 post-infection animals from group 2 were treated with TCZ. At 122 dpi animals were transported and fed with high-energy diet at the intensive animal farming facility until slaughter at the abattoir.

2.2. Collection of samples

Animals were bled for complete hemogram analysis before the infection (day 0) and after 43, 87, 157 and 213 dpi. Faecal and blood samples were collected before (day 0) and at 15, 28, 43, 59, 71 and 87 dpi (every approximately 15 days) and at 115, 157, 193 and 213 dpi (every approximately 30 days) during the acute and chronic phases of infection, respectively (Figure 1). *F. hepatica* egg counts per gram (EPG) in faeces were determined individually using the

158 sedimentation technique (32) before the infection (day 0) and at 15, 28, 43, 59, 71, 87, 115,
159 157, 193 and 213 dpi. Chronic infection was determined after 15 weeks of parasite infection.
160
161 *2.3. Fluke Recovery and liver damage*
162 Livers, bile ducts and gallbladders were examined for the presence of *F. hepatica* parasites as
163 previously described (34). Fluke recovery was determined by the number of adult flukes from
164 each processed liver. Livers were weighed and given a score according to the macroscopic liver
165 damage (ranging from 0 to 3) at the time of dissection by a veterinary pathologist, where 0
166 represented absence of tissue necrosis and liver damage, 1 represented less than 30% (slight),
167 2 between 30 and 70% (moderate), and 3, more than 70% (severe) of liver necrosis and damage
168 at the tissue surface. Fibrosis, capsule, consistency, calcification in the biliary ducts and
169 abscesses, as well as atrophy of hepatic lobes were also determined by a similar score: 0
170 (absence), 1 (slight), 2 (moderate) and 3 (severe) (35).
171
172 *2.4. Hemogram and circulating leukocyte counting*
173 Blood samples were processed for assessment of haematocrit mean corpuscular volume (MCV)
174 and mean platelet volume (MPV) using the Counter 19 from Weiner lab. Leukocyte total counts
175 were determined in a microscope using a Neubauer Haemocytometer. Thin smears were
176 prepared on individually labelled microscope slides using one or two drops of blood. Smears
177 were air-dried, fixed with absolute methanol, and stained with Giemsa to analyse leukocyte and
178 lymphocyte counts. Sera were collected for the quantification of transaminase activity levels.
179
180 *2.5. Hepatic synthetic functions and transaminase determination*
181 Serum levels of albumin, total protein and hepatic enzyme activity were determined using an
182 automatized spectrophotometer (Dimension RxL Max integrated chemistry system; Siemens).
183 Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Gamma

184 glutamyltransferase (GGT) activities were expressed as international units per litre (IU/l), Total
185 Bilirubin (TBil) and Direct Bilirubin (DBil) determinations were expressed in mg/dl and
186 Albumin (ALB) and Total Protein (TP) determinations were expressed in g/dl.
187
188 *2.6. Statistical analysis*
189 Results were analysed using GraphPad Prism software 6.0 (GraphPad Software, San Diego,
190 CA) by non-parametric (with Krustal-Wallis test) or parametric one- or two-way ANOVA
191 followed by the Tukey test for multiple comparisons, according to the experiment. Asterisks
192 represent statistically significant differences as follows: * $p < 0.05$, **$p<0.01$, ***$p<0.001$ and
193 ****$p<0.0001$.

3. Results

3.1. Characterization of acute and chronic stages of F. hepatica experimental infection in steers

To study and characterize the local and physiopathological effects during both acute and chronic *F. hepatica* infection in cattle, we experimentally infected steers with 500 *F. hepatica* metacercariae per animal. After experimental infection, animals showed an increase in faecal EPG from day 87, although this increase was significantly higher only after 115 dpi (Figure 2A). As expected, non-infected animals did not display detectable EPG in faeces during the whole experiment (Figure 2A). Interestingly, the increase in EPG in *F. hepatica*-infected animals varied during the infection period: sustained faecal EPG were constant between days 115 and 193, while they decreased at day 213, although they remained significantly increased with respect to control steers (Figure 2A).

The number of recovered flukes in the livers from infected animals analysed at slaughter was heterogeneous, with a mean of 89 flukes per liver from infected steers, while non-infected cattle did not show any flukes in livers (Figure 2B). Moreover, only between 5 to 40% of flukes were recovered in infected animals with respect to the initial number of metacercariae inoculated in these animals (Figure 2C). Last, the number of recovered flukes was significantly associated with faecal EPG at day of slaughter (Figure 2D). Fluke recovery number was also significantly correlated with liver damage (Figure 2E).

Last, livers from infected animals were characterized (Figure 2F and G) by a high degree of fibrosis, right lobe hypertrophy, hyperplasia of bile ducts and a pale colour comparing to livers from control animals (Figure 2H and I, and Table I).

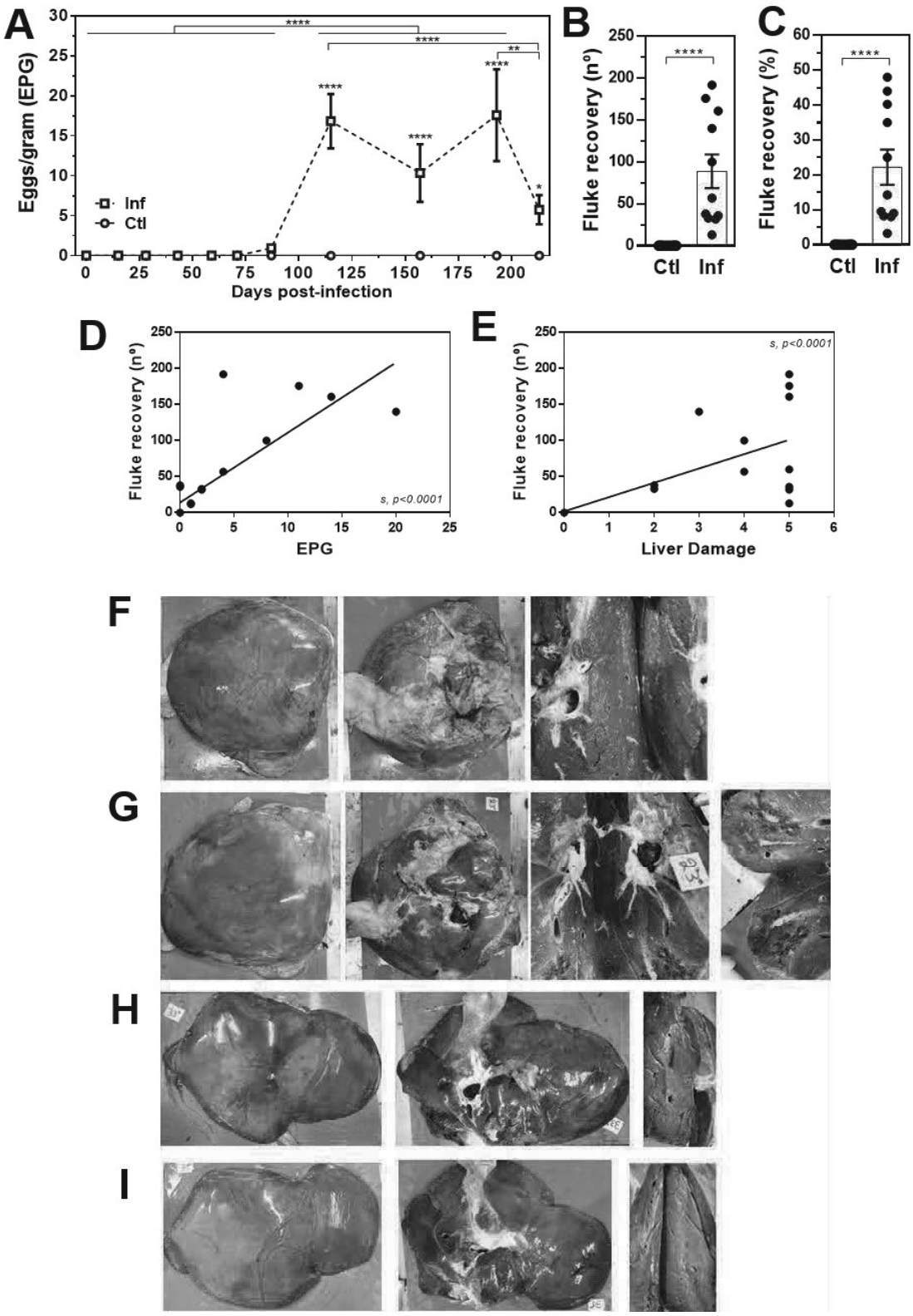

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Figure 2. Fluke recovery from *F. hepatica* -infected animals significantly correlates with faecal parasite eggs/gram (EPG). A) EPG in faeces from infected (squares) and non-infected animals (circles). Animals were orally infected with 500 metacercariae and EPG were determined by the sedimentation technique. Only the infected group and control groups are shown (n=12). B-C) Fluke recovery from infected cattle at time of sacrifice both in number of flukes (B) or percentage in relation to the initial number of metacercariae used to infect animals (C). Asterisks indicate statistically significant differences between infected and control animals calculated with two-way Anova followed by a Tukey multiple comparison test (A) or non-parametric one-way Anova with Krustal-Wallis test (B-C): *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. D-E) Significant linear regression correlation between fluke recovery and EPG (D) and liver damage (E). F-G) Representative livers from infected that had 13 (F) and 57 (G) flukes in the liver. H-I) Representative livers from control animals.

Table I. Macroscopic characteristics of livers from experimentally infected steers.

Groups	Score	LD	F	RLH	LLA	HBD	RB	CT	Cal	Con
INF	0	0 (0)	0 (0)	2 (17)	0 (0)	0 (0)	2 (17)	2 (17)	1 (8)	1 (8)
	1	1 (8)	1 (8)	0 (0)	3 (25)	2 (17)	0 (0)	4 (33)	1 (8)	0 (0)
	2	2 (17)	3 (25)	6 (50)	4 (33)	1 (8)	8 (67)	6 (50)	4 (33)	3 (25)
	3	9 (75)	8 (67)	4 (33)	5 (42)	9 (75)	2 (17)	0 (0)	6 (50)	8 (67)
INF /TCZ	0	0 (0)	2 (17)	8 (67)	0 (0)	1 (8)	7 (58)	7 (58)	5 (42)	5 (42)
	1	9 (75)	1 (8)	1 (8)	1 (8)	5 (42)	2 (17)	3 (25)	2 (17)	1 (8)
	2	2 (17)	6 (50)	3 (25)	7 (58)	4 (33)	2 (17)	2 (17)	3 (25)	3 (25)
	3	1 (8)	3 (25)	0 (0)	4 (33)	2 (17)	1 (8)	0 (0)	2 (17)	3 (25)
Control	0	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)
	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

The number of animals together with the % (between brackets) is indicated for each score: n (%). LD: liver damage; F: fibrosis; RLH: right lobe hypertrophy; LLA: left lobe atrophy; HBD: Hyperplastic bile ducts; RB: round borders; CT: Capsule thickening; Cal: calcification; Con: consistency.

3.2. Increased levels of serum AST and GGT are detected in early stages of infection

To analyse the hepatic synthetic function of infected animals, we studied the production ratio between albumin and total protein, bilirubin, and different transaminase activities in sera from infected and control steers. Infection was associated with a decrease of albumin/total protein production from 71 to 115 dpi (Figure 3A). These differences became clearer when the albumin/total protein production ratio by the liver was normalized to control animals (Figure 3B). Direct bilirubin levels were significantly increased both in the acute and early chronic stage of the infection in relation with non-infected steers (Figure 3C). Of note, a statistical difference was detected on bilirubin levels in both groups along the infection, probably due to seasonal or nutritional changes related with the maintenance of animals.

In order to expand the study of the pathological effect induced by *F. hepatica* in bovine livers, we also analysed AST, ALT and GGT activities in sera from infected and control animals. As indicated in Figures 3D to 3F, an increase of the three studied enzyme activities in sera of infected animals was detected in relation to sera from control steers. However, the increase was different for the three analysed enzyme activities. AST activity in sera was increased in infected steers both in the acute and chronic phases of infection (between 28 and 157 dpi) with regard to control animals (Figure 3D). On the other hand, ALT activity significantly increased only between 71 and 87 dpi (Figure 3E). Last, GGT activity in sera of infected animals increased at day 71 after infection, as ALT, although it continued significantly increased until animal slaughter (Figure 3F). AST enzymatic activities in serum significantly correlated with both faecal EPG and fluke recovery in livers (Figure 3G).

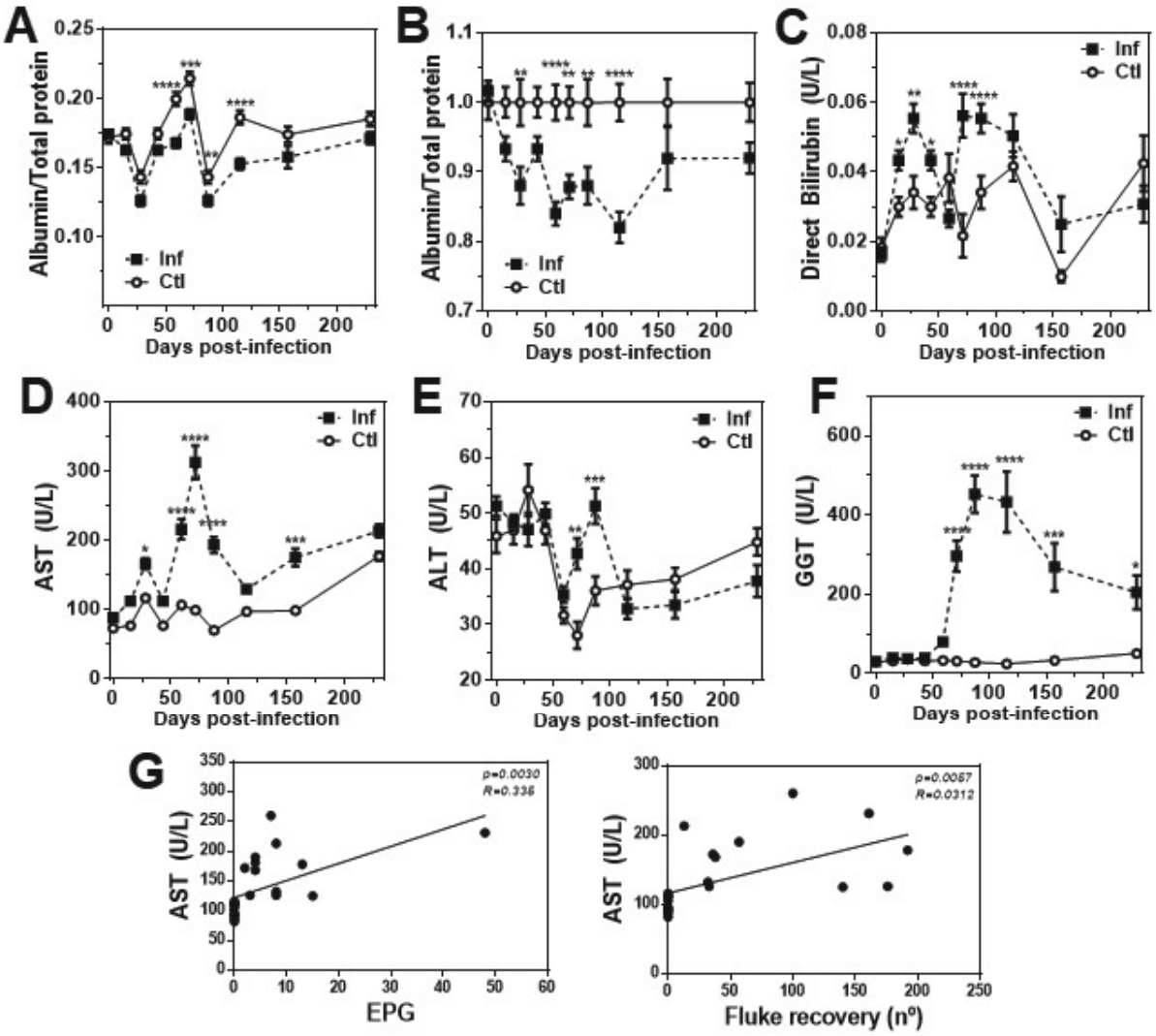

Figure 3. Infected animals present a compromised liver function and hepatic damage. A) Albumin and total protein ratio determined in U/L in sera of infected and control cattle. B) Normalised albumin and total protein ratio in regard to the control group of infected and control cattle. C) Direct Bilirubin detection in U/L of infected and control cattle. D) aspartate transaminase (AST) serum levels. E) Alanine transaminase. (ALT) serum levels. F) Gamma-glutamyl transferase (GGT) serum levels. Only the infected group and control groups are shown (n=12). Asterisks indicate statistically significant differences between infected and control animals calculated with two-way Anova followed by a Tukey multiple comparison test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. G) Significant linear regression correlation between AST levels and EPG (left) at day 157 post-infection or fluke recovery (right).

255

256 *3.3. High circulating eosinophil counts are correlated with fluke number in livers from infected*

257 *cattle*

Considering the hematophagous characteristic of the parasite, we analysed red blood cells and haemoglobin from infected and control groups of animals. From a general point of view red blood cell number (RBC), total haemoglobin (Hb) and haematocrit (HCT) remained constant during the infection in steers, although a significant increase in HCT was detected at 87 dpi (Figure 4A to C), revealing no signs of parasite associated anaemia. Other related parameters such as mean platelet volume (MPV) or mean corpuscular volume (MCV) of red blood cells did not change during the course of the infection (not shown). On the other hand, platelet number (PLT) and plateletcrit (PCT) increased in infected animals during the course of infection (Figure 4D and E, respectively), while mean platelet volume (MPV) and platelet distribution width (PDW) did not show any significant difference between infected and control steers (Figure 4F and G, respectively). Of note, only fluke recovery number, and not faecal EPG, was significantly associated with PCT (Figure 4H). These results indicate that an increase in platelets is related with *F. hepatica* infection, which could trigger liver fibrosis and regeneration (36).

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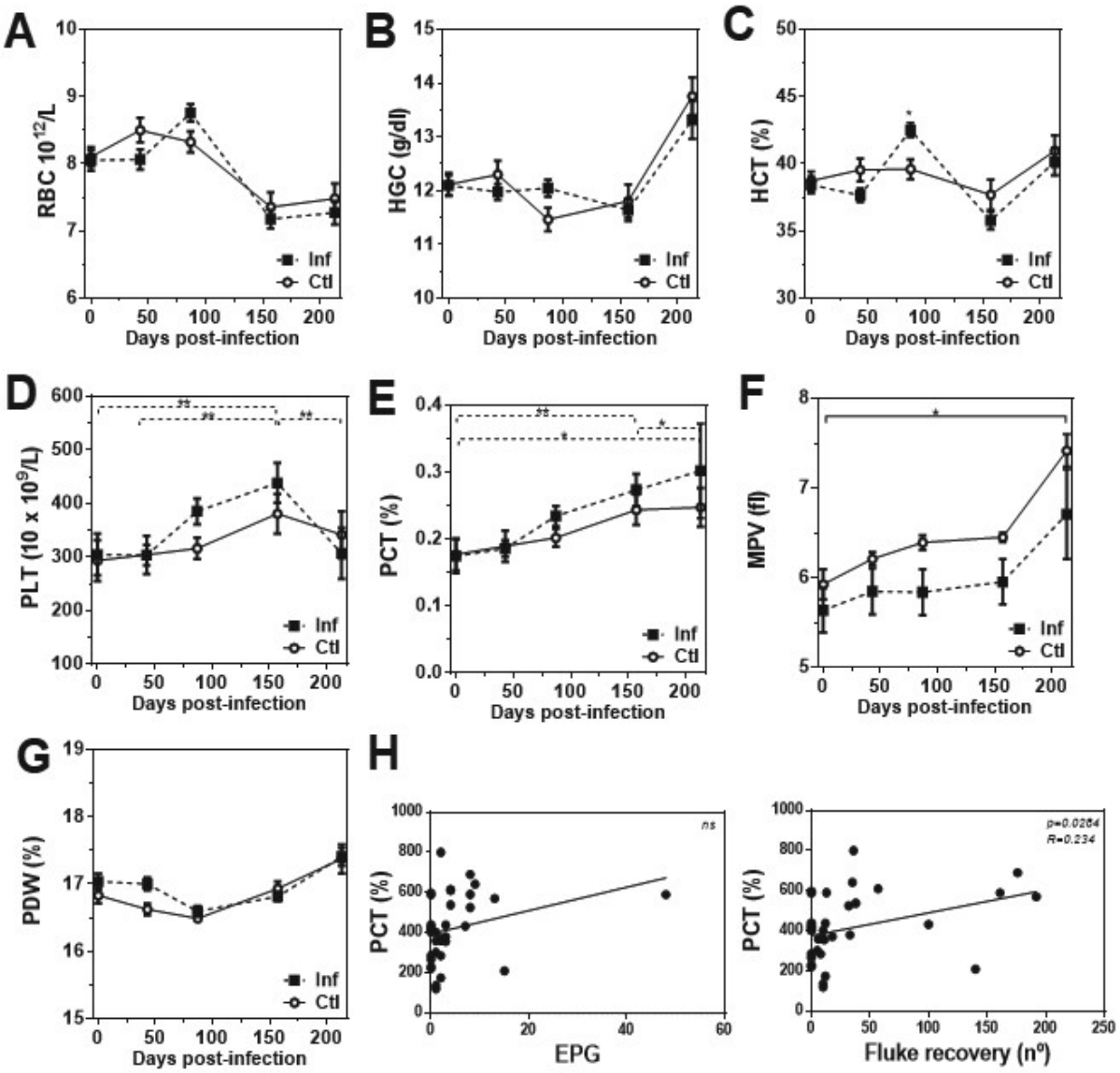


Figure 4. Infected animals do not present any signs of anaemia associated with *F. hepatica* infection while platelet number and plateletcrit increase during *F. hepatica* infection. A-C) Red blood cells (RBC, A), total haemoglobin (Hb, B) and haematocrit (HCT, C) were determined using an automated counter in infected and control groups of steers. E-G) Platelet (PLT, D), Plateletcrit (PCT, E), Mean Platelet Volume (MPV, F) and Platelet distribution width (PDW, G) were determined using an automated counter in infected (black squares) and control groups (open circles) of animals. Dotted and continuous lines indicate significant differences between different time points in the infected and control groups, respectively. Only the infected group and control groups are shown (n=12). Asterisks indicate statistically significant differences between infected and control animals calculated with two-way Anova followed by a Tukey multiple comparison test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. H) Significant linear regression correlation between PCT (%) and EPG (left) at day 157 post-infection or fluke recovery (right).

Steers presented similar levels of circulating leukocytes during experimental *F. hepatica* infection, although, interestingly, in the acute and early chronic stages of the infection control animals increased their number while those from infected steers did not change over time (Figure 5A). A similar result was found for blood lymphocytes, which decreased at 49 dpi in infected steers (Figure 5B). On the other hand, monocytes and neutrophils augmented after day 87 in the non-infected group of animals, while infected steers remained stable throughout the whole infection period (Figure 5C and D, respectively). Last, granulocyte frequency, such as basophils or eosinophils in circulation significantly increased upon infection. With regard to basophils, infected animals presented two picks: one at the acute and the other at the chronic phases of infection, while non infected animals remained unchanged during the period of study (Figure 5E). As expected, eosinophils largely increased from 48 dpi remaining high during both the acute and early chronic phases of the infection (Figure 5F). Nevertheless, we could not detect any difference between the frequency of eosinophils from infected and control animals at day of slaughter (Figure 5F). On the other hand, although no significant correlation between the frequency of circulating eosinophils and faecal EPG was detected at 43 dpi, there was a significant positive correlation between the frequency of eosinophils at the acute phase of the infection and fluke recovery number in livers (Figure 5G).

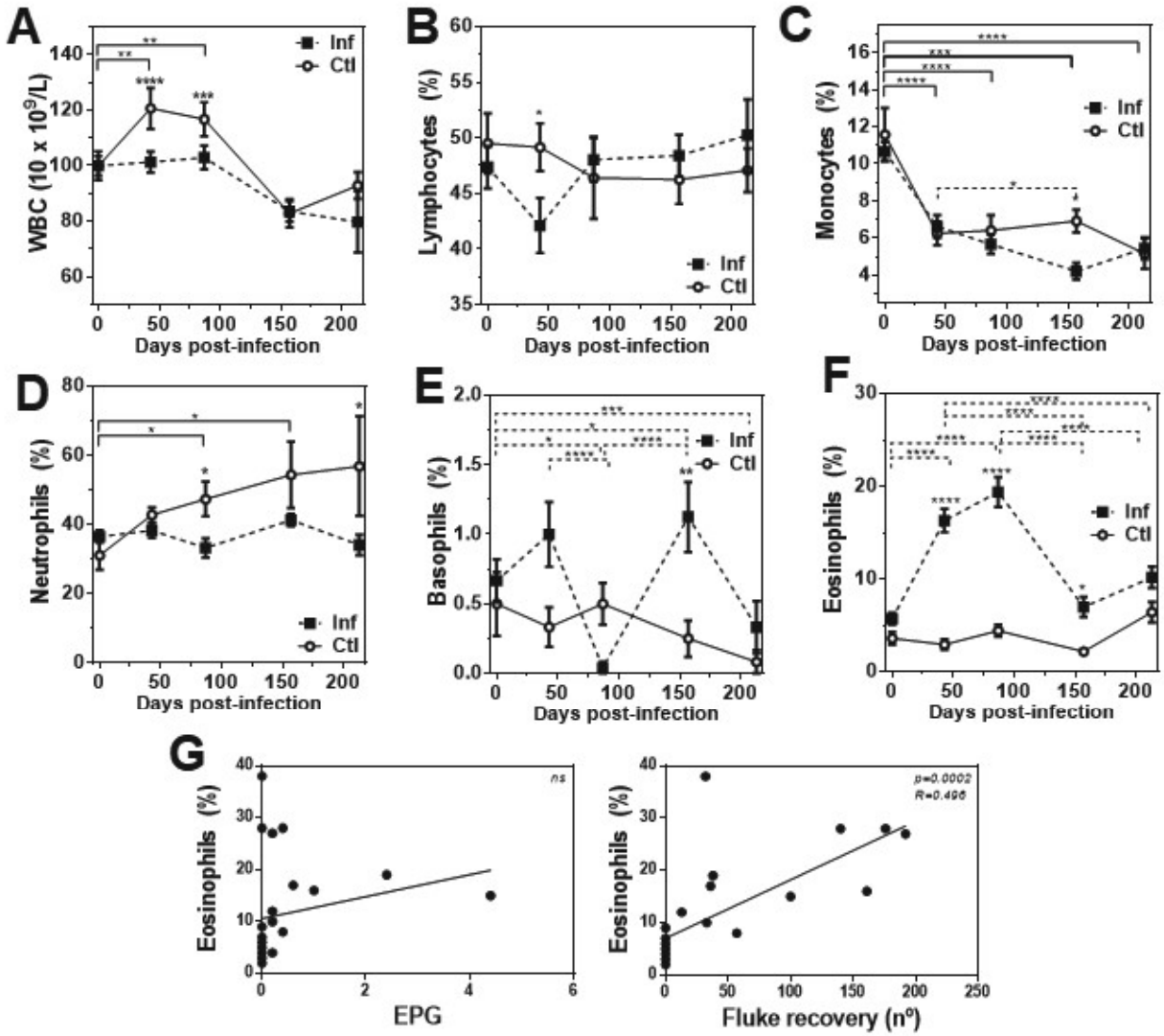


Figure 5. *F. hepatica* infection is associated with a decrease in white blood cells but an increment in eosinophils. White blood cells (WBC, A), lymphocytes (B), monocytes (C), neutrophils (D), basophils (E) and eosinophils (F) in blood were detected in blood smears prepared on individually labelled microscope slides and stained with Giemsa. Dotted and continuous lines indicate significant differences between different time points in the infected and control groups, respectively. Asterisks indicate statistically significant differences between infected and control animals calculated with two-way Anova followed by a Tukey multiple comparison test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. G) Linear regression correlation between eosinophils (%) and EPG (left) at 43 dpi or fluke recovery (right).

290

291 *3.4. Treatment with TCZ does not completely eliminate hepatic flukes or abrogates liver*
292 *damage*

293 In order to evaluate the effect of a commonly used anti-helminth drug on parasite infection as
294 well as on liver damage and dysfunction, 12 out of the 24 infected steers were treated with TCZ
295 in the chronic phase of *F. hepatica* infection (at 115 dpi). The TCZ-treatment induced, as
296 expected, a considerable decrease in faecal EPG from 157 dpi with regard to infected animals
297 (Figure 6A), which remained unchanged as the control group until the end of the study. TCZ-
298 treated *F. hepatica* infected animals showed a reduction between 10 and 15% of faecal EPG in
299 comparison with the initial number of EPG before TCZ treatment (Figure 6B). However, TCZ-
300 treatment of infected steers did not completely eliminate all flukes in the biliary tracts of livers
301 since a significant increase in fluke recovery (6 to 35 flukes) was determined in comparison to
302 control animals, although their number was significantly reduced compared to the non-treated
303 *F. hepatica* steers (32 to 192 flukes) (Figure 6C and Table II). Fluke recovery in infected steers
304 varied from 3 to 38% in relation to the initial inoculation of 500 metacercariae (100%), while
305 TCZ-treatment of infected animals reduced this number to 1 to 7% (Figure 6D and Table II).
306 However, the efficiency of TCZ-treatment was from 5 to 39% taking into account the mean of
307 fluke recovery from infected animals (Figure 6E and Table II).
308
309 **Table II**. Fluke recovery from *F. hepatica*-infected steers with or without TCZ-treatment.

	Fluke recovery (nº)	**Fluke recovery (%)**	**Fluke recovery (%) for TCZ efficiency***
F. hepatica	88.1 (32-192)	17.8 (3-38)	100 (15-198)
***F. hepatica* + TCZ**	12.3 (6-35)	2.5 (1-7)	13.8 (5-39)

310 **To calculate TCZ efficiency the nº of flukes (88.1, column 1) was considered as 100% (column 3).*

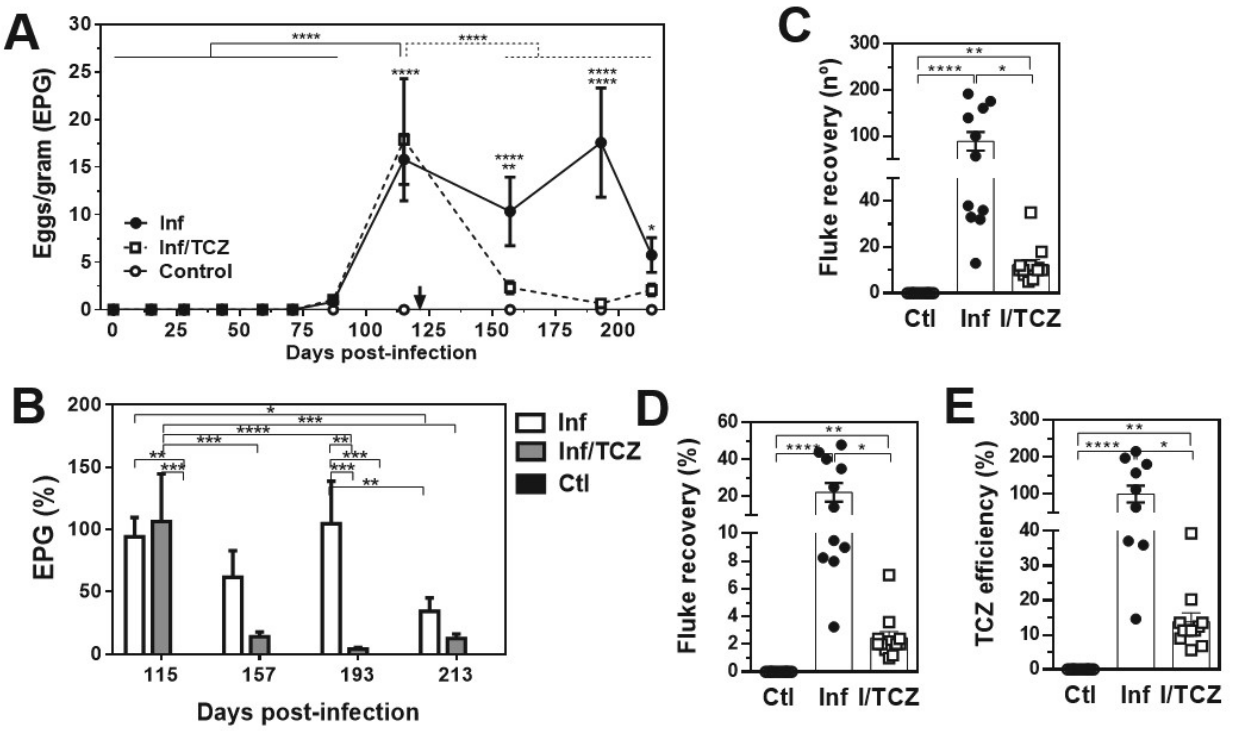


Figure 6. Treatment with triclabendazole (TCZ) decreases fluke recovery although does not completely eliminate hepatic damage. A) EPG from infected (close squares), TCZ-treated infected (open squares) and non-infected animals (open circles). Animals were orally infected with 500 metacercariae and EPG were determined by the sedimentation technique. The arrow indicates the moment of TCZ administration (115 dpi). B) EPG percentage from infected cattle with or without treatment with TCZ. Number of recovered flukes from livers of infected cattle with or without TCZ-treatment at time of sacrifice (213 dpi). D) Percentage of recovered flukes considering the initial number of metacercariae administrated as 100% (500). E) TCZ efficiency calculated with respect to non-treated infected animals. The mean of recovered flukes from infected animals was considered as 100% (88.1 flukes). Infected group, TCZ-treated/infected animals and control groups are shown (n=12/group). Asterisks indicate statistically significant differences between infected and control animals calculated with non-parametric two-way Anova followed by a Tukey multiple comparison test (A and B) or non-parametric one-way Anova with Krustal-Wallis test (C-E): *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

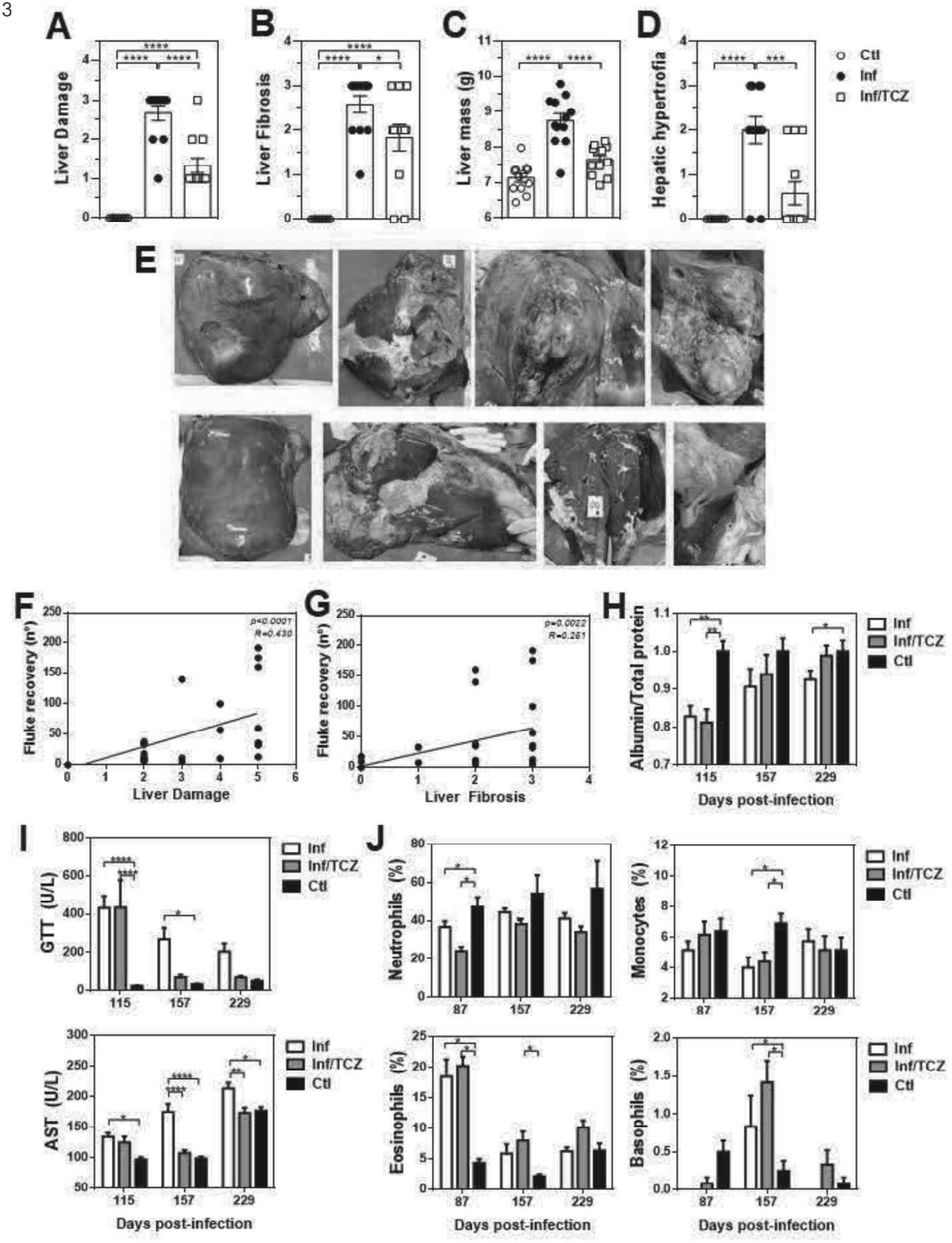
In order to analyse the type and degree of liver lesions associated with TCZ-treatment of *F. hepatica*-infected steers, we developed a score to quantify damage, fibrosis and lobe hypertrophy, as explained above. Infected animals showed significantly higher hepatic damage (Figure 7A) and fibrosis (Figure 7B) than controls. TCZ-treated infected animals also showed higher levels of liver damage and fibrosis than control steers, although they were lower than non-treated animals (Figure 7A and B). Of note, TCZ-treatment completely abrogated the increase of liver mass (Figure 7C) and right hepatic lobe hypertrophy (Figure 7D) induced by

the infection (Figure 7E and Table I). Interestingly, both liver damage (Figure 7F) and fibrosis (Figure 7G) were significantly correlated with the number of recovered flukes.

Last, we analysed how the hepatic function and circulating leukocytes were affected by TCZ-treatment of infected animals. As shown in Figure 7H, TCZ-treated animals did not show any significant differences in the albumin/total protein ratio in comparison with non-treated infected animals. However, only infected animals showed a significant decrease in albumin/total protein levels compared with the control group (Figure 7H). In addition, both GGT and AST activity levels in serum from infected animals remained significantly higher than TCZ-treated infected and control groups (Figure 7I). Finally, no significant changes in circulating neutrophils, monocytes, eosinophils or basophils were associated with TCZ-treatment of infected animals (Figure 7J). Altogether these results indicate that, although TCZ-treatment reduces parasite burden and damage in the liver while it is not able, however, to completely avoid them.

Figure 7 in next page

Figure 7. TCZ-treated cattle present liver damage and liver fibrosis, which correlates with fluke recovery. Liver damage (A), fibrosis (B) and right lobe hypertrophy (C) was determined by a veterinary anatomopathologist anatomic. Livers were also weighted (D). Significant linear regression correlation between fluke recovery and liver damage and EPG (E) or fibrosis (F) of both infected and infected/TCZ treated groups. G) Albumin and total protein ratio determined in U/L in sera from *F. hepatica* infected animals with or without TCZ-treatment and control steers at 115, 157 and 213 dpi. H) Gamma-glutamyl transferase (GGT) and aspartate transaminase (AST) plasmatic levels. Percentage of neutrophils, monocytes, basophils and eosinophils (I) in blood at 115, 157 and 213 dpi were detected in blood smears prepared on individually labelled microscope slides and stained with Giemsa. Infected group, TCZ-treated/infected animals and control groups are shown (n=12/group). Asterisks indicate statistically significant differences between infected and control animals calculated with one-way Anova (A-D) or two-way Anova followed by a Tukey multiple comparison tests (G-I): *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. J-K) Representative livers from TCZ-treated infected animals that had 10 (J) and 11 (K) flukes in the liver.


A
Liver Damage
B
Liver Fibrosis
C
Liver mass (g)
D
Hepatic hypertrofia
Ctl
Inf
Inf/TCZ
E
F
Fluke recovery (n°)
p<0.0001
R=0.430
Liver Damage
G
Fluke recovery (n°)
p=0.0022
R=0.261
Liver Fibrosis
H
Albumin/Total protein
Days post-infection
I
GTT (U/L)
AST (U/L)
Days post-infection
J
Neutrophils (%)
Monocytes (%)
Eosinophils (%)
Basophils (%)
Days post-infection

4. Discussion

This work deeply characterized liver function markers. haematological and circulating leukocyte dynamics in 30 weeks of an experimental *F. hepatica* infection in steers, together with the impact of TCZ-treatment in liver damage during the course of chronic experimental parasite infection.

Faecal EPG from infected animals varied during the infection process and only started to significantly increase after 16 weeks from infection (wpi), while it decreased at time of slaughter (30 wpi). It is already known that faecal EPG, although used as the gold standard assay, has a low sensitivity in cattle since it can only detect patent infection and also because egg shedding is discontinuous (21, 37). However, it is cost effective, simple and does not require specialized instrumentation (38, 39). Surprisingly, previous reports in cattle indicate that faecal EPG can be detected from 10–11 wpi (40), much earlier than what it was found in our experimental infection (16 wpi). We also found great heterogeneity in EPG and recovered flukes from livers in the infected group, although infections were carefully handled and administration of metacercariae in capsules was successful. Thus, altogether, these facts highlight the drawbacks of coprological methods for fasciolosis diagnosis (38) and the need of alternative diagnostic methods. Indeed, an integral diagnostic can be more reliable, especially those that combine both coprological and immunological methods including antigen detection and serological assays (38, 41, 42).

The heterogeneity found in faecal EPG was also correlated with fluke recovery from livers from infected animals demonstrating that individual genetic background of selected animals and/or the experimental infection procedure might affect parasite survival in the infected animals. This fact is relevant, considering that we analysed an experimental infection with minimal

364 variability, as compared to natural infections, in which cattle might be permanently challenged
365 with metacercariae present in the pastures, and where acute or chronic phase of the disease is
366 difficult to determine since immature versus mature fluke coexist in cattle (31).
367
368 Both protein and bilirubin determination in sera allowed the assessment of hepatic dysfunction
369 in protein synthesis and possible cholestasis in the livers of infected animals, especially in the
370 acute (until 10 wpi) and early chronic phases of the infection (from 10 to 16 wpi). However,
371 these differences were not detected after 16 wpi, indicating that liver regeneration can take place
372 to supply adequate protein levels. Interestingly, infected-steers presented an increase in both
373 platelet number and plateletcrit at 22 wpi which could be promoting liver fibrosis and
374 regeneration in the chronic phase of *F. hepatica* infection. Indeed, it has already been
375 demonstrated that platelets improve liver regeneration directly on hepatocytes, although their
376 role in liver diseases is still controversial (36).
377
378 This study also demonstrates that the analysis of different transaminase activities in sera from
379 *F. hepatica*-infected animals reveal different increased kinetic profiles, as already suggested in
380 previous studies (26-28). The different profile obtained for the studied transaminases may be
381 related with their organ-specific expression or function according to the hepatic dysfunction or
382 biliary tract obstruction. In fact, ALT is the only enzyme produced by hepatocytes, being more
383 specific than AST and GGT and results to be a very specific marker of hepatocellular injury
384 (43). However, its expression can fluctuate during the day and according to the level of
385 strenuous exercise (43). This latter fact might explain the differences obtained for both infected-
386 and control groups of animals during the studied period of time. On the other hand, AST and
387 GGT can be produced by other organs, such as the kidney, apart from the liver. Nevertheless,
388 AST usually rises in conjunction with ALT to indicate hepatocellular injury, while high serum

GGT activity suggests biliary tract obstruction (cholestasis) (43). Furthermore, this study indicates that *F. hepatica* infection was associated with an early increase (at 4 wpi) of AST in sera, while both GGT and ALT increased levels were detected after 10 wpi suggesting hepatic dysfunction likely due to liver damage in the parenchyma induced by the juvenile flukes, which can be observed up to 6 wpi (5). On the other hand, increased levels of GGT were detected after 10 wpi and lasted the whole period of infection (up to 30 wpi), indicating cholestasis associated with the chronic phase of infection. Importantly, a recent report using a transcriptomic approach analysing immune responses in peripheral blood mononuclear cells of experimentally infected cattle demonstrated that gene pathways for hepatic fibrosis and cholestasis were enriched at chronic stages (16). Moreover, the hepatic damage of experimental infected cattle was associated with excessive reactive oxygen species production (30). Altogether, these results suggest that AST could be used to detect acute stages of infection in experimental infected-cattle, which turns out to be at least as earlier as antigen detection or serological tests. These last methods are capable of diagnosing fasciolosis between 2 and 4 wpi (23, 38, 44, 45) or 2 wpi, respectively (38, 44, 46). In addition, fasciolosis acquired by natural infection in different mammalian hosts was also associated with increase of hepatic transaminases, although an association with the stage of the infection was difficult to determine (31, 47-49). Thus, the use of ALT, AST and GGT activity levels in plasma to detect natural infection may be limited likely due to the coexistence of immature and mature flukes in the livers of subclinical infected cattle.

Eosinophils participate mainly in the defence against multicellular parasites and in several Th2-driven immune disorders (50). The classical functions of eosinophils include mainly degranulation triggered by antibodies in a mechanism known as antibody-dependent cell cytotoxicity (50, 51). Their functions have been well characterized in helminth infections, and in particular, our group has recently demonstrated that they play a protective role during

414 experimental *F. hepatica* infection in mice (52). The fact that an increase in the frequency of
415 circulating eosinophils was detected in the acute phase of infection (at 4 wpi) and that they were
416 significantly correlated with fluke recovery number, but not with EPG, at 43 dpi, indicates that
417 they would be more useful and reliable to detect early stages of the experimental infection,
418 together with other hepatic markers. It also confirms the fact that EPG is a late and likely a non-
419 confident technique to follow infection, as it has been previously reported (53, 54). Thus, our
420 experimental study provides case-control groups and establishes a better association between
421 liver pathological changes and serum biochemical alterations in *F. hepatica* experimental
422 infection in cattle during a long period of time (30 wpi) (55, 56). However, immunological
423 studies including humoral and cellular immune response elicited by *F. hepatica* should be
424 further investigated to complement the circulating leukocyte population dynamics performed
425 in this work.
426
427 It is worth noting that steers, regardless of *F. hepatica* infection, presented some variations in
428 some of the red or white blood counts during the analysed period of time, although they
429 remained in general between values of reference (57). This indicates that animals might be
430 sensitive to other factors, independently of parasite infection. These may include seasonal or
431 nutritional changes, variation in the distribution of eggs within a single faecal specimen, daily
432 fluctuations of faecal production and consistency in the host (40, 44, 57), animal age or other
433 factors influenced by the environment shared by the animals. Indeed, steers were free grass-fed
434 up to day 110 after the infection and then transported to a feedlot facility where they were
435 intensively feed with high energy diets. After transport, animals lost some weight (not shown).
436 Therefore, both the transportation-induced stress as well as the nutritional changes during the
437 experiment might have influenced the synthetic liver function and circulating blood and white
438 cells, regardless of parasite infection.

TCZ is one of the antihelminthics for the treatment of fasciolosis, with a mechanism of action that involves disruption of the parasite tegument and causes severe damage to the reproductive system of *Fasciola* spp. (25, 58). Although effective, the increase of TCZ-resistant flukes in different parts of the world is an important drawback (17, 25, 59). TCZ-treatment has shown to be effective, especially during the acute phase of fasciolosis, since it would prevent liver damage. Indeed, in recent studies the efficacy of TCZ was confirmed in cattle (59) but mainly in sheep and goats (19, 60, 61), although the histopathological effects caused by the parasite in the liver were not studied in depth during the chronic stage of the infection. Indeed, one previous work analysed the serological and coproantigen ELISA and EPG in cattle experimentally infected with *F. hepatica* until 126 dpi with TCZ treatment, although no hepatic lesions were described (20). Interestingly, the authors found that steers infected with 500 metacercarie and treated at 84 dpi did not have detectable EPG or flukes in the liver (20). However, our results strikingly demonstrate that the administration of TCZ at 115 dpi in steers infected with 500 TCZ-sensitive metacercariae, although significantly reduced parasite burden in the liver, did not kill all the flukes in the liver, a fact that was accompanied by considerable hepatic damage. However, it remains to be determined whether TCZ treatment was associated with the presence of immature flukes in the livers of infected animals. Of note, animals treated with TCZ did not show any detectable faecal parasite eggs, likely due to the low sensitivity of the technique. An important issue is that in our study steers were maintained in snail-free establishments, preventing the parasite to continue its life cycle in the intermediate host. Thus, TCZ-treatment in the chronic phase of infection was associated with significant hepatic damage and would not resolve the economic losses due to confiscation of livers. In fact, condemnation of *Fasciola*-infected bovine livers at slaughter represents a significant loss of income for livestock in the world (1), including Uruguay (11).

5. Conclusions

In conclusion, our work sheds light into the physiopathological parameters associated to both acute and chronic phases of fluke experimental infection in cattle, revealing the complexity of the host response to the infection, together with the effects of TCZ-treatments in chronically infected animals.

Declaration of interest

The authors declare no conflict of interests.

Animal welfare statement

Animal experimentation was carried out according to the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences.

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Author contributions

Monique Costa: Investigation, data curation and analyses, original draft of the manuscript; Anderson Saravia: Biological sample collection and parasitology and reviewing the manuscript; Diego Ubios: Animal care and biological sample collection; Pablo Lores, Valeria da Costa, María Florencia Festari, Mercedes Landeira and Santiago A. Rodríguez-Zraquia: data curation and analyses and reviewing the manuscript; Georgget Banchero: Conceptualization and reviewing the manuscript. Teresa Freire: Conceptualization, experiment design and supervision, analyses of data and writing, review and editing the manuscript.

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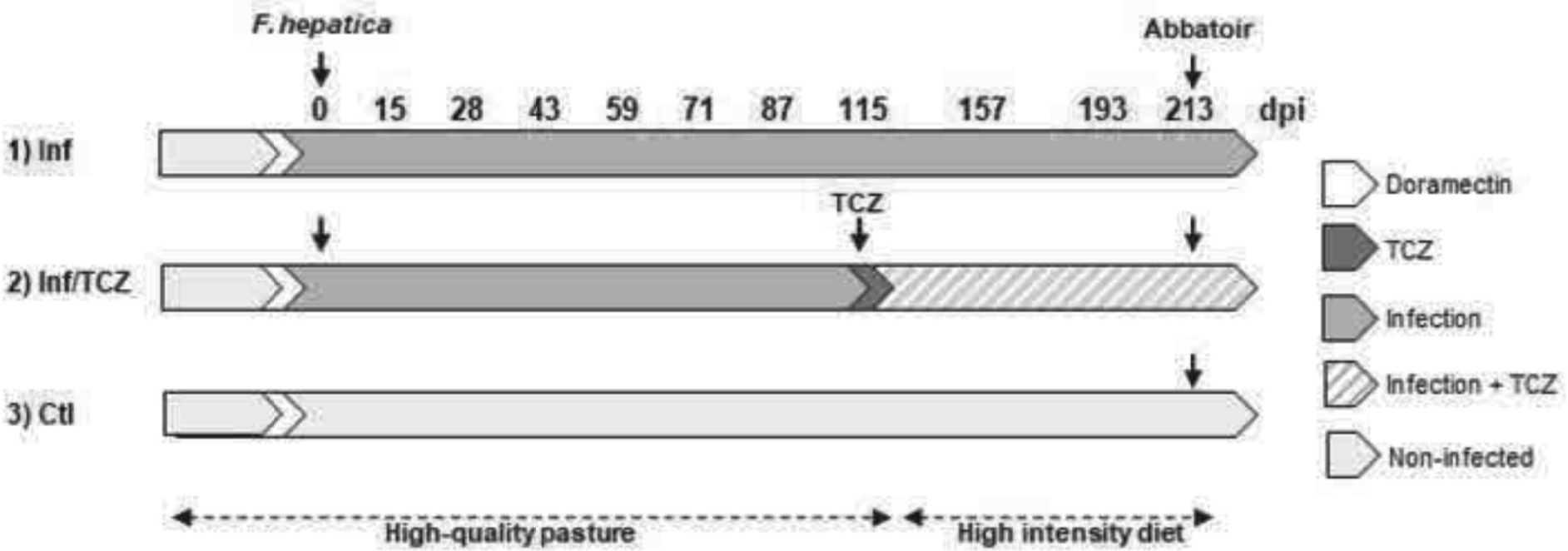
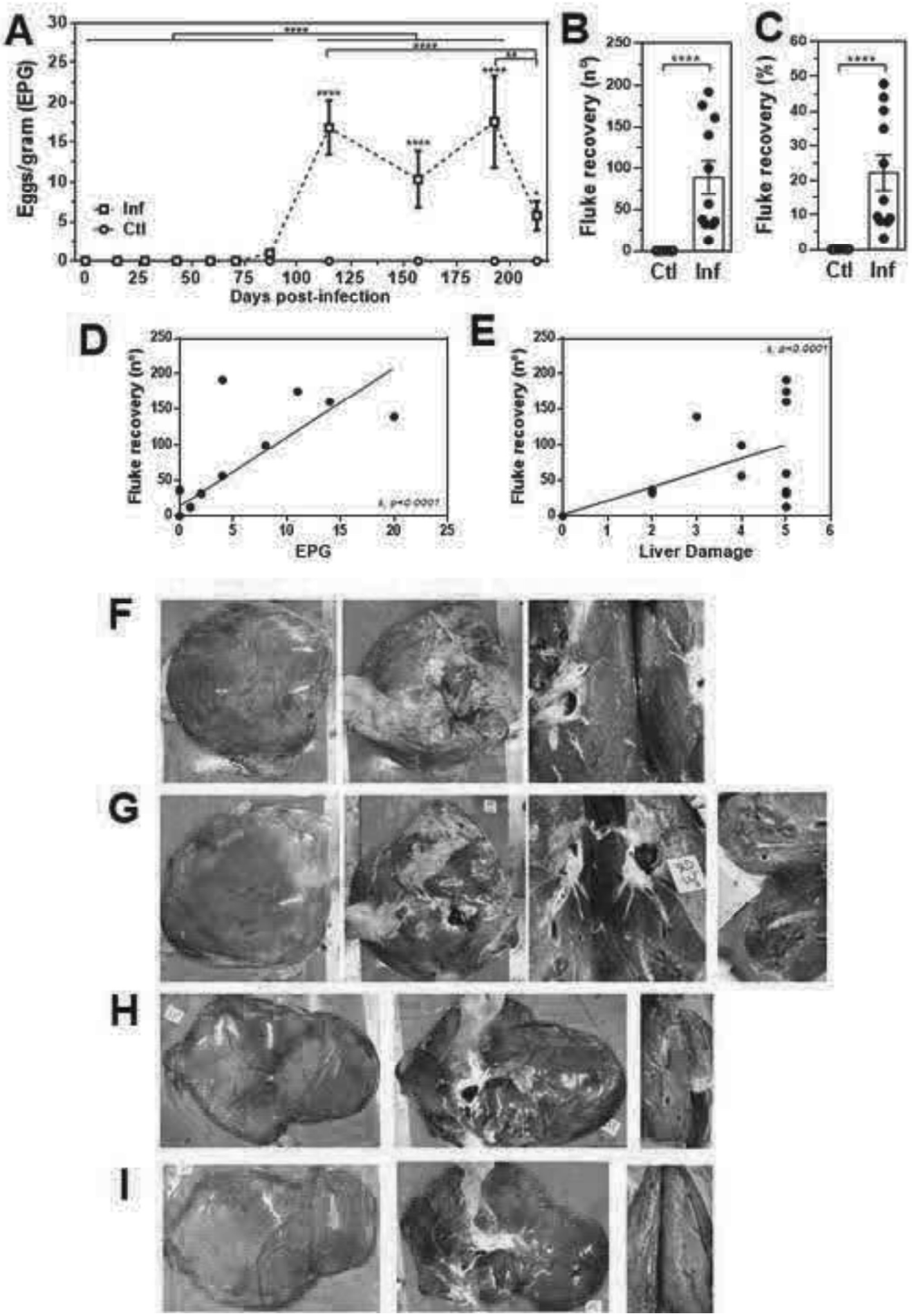
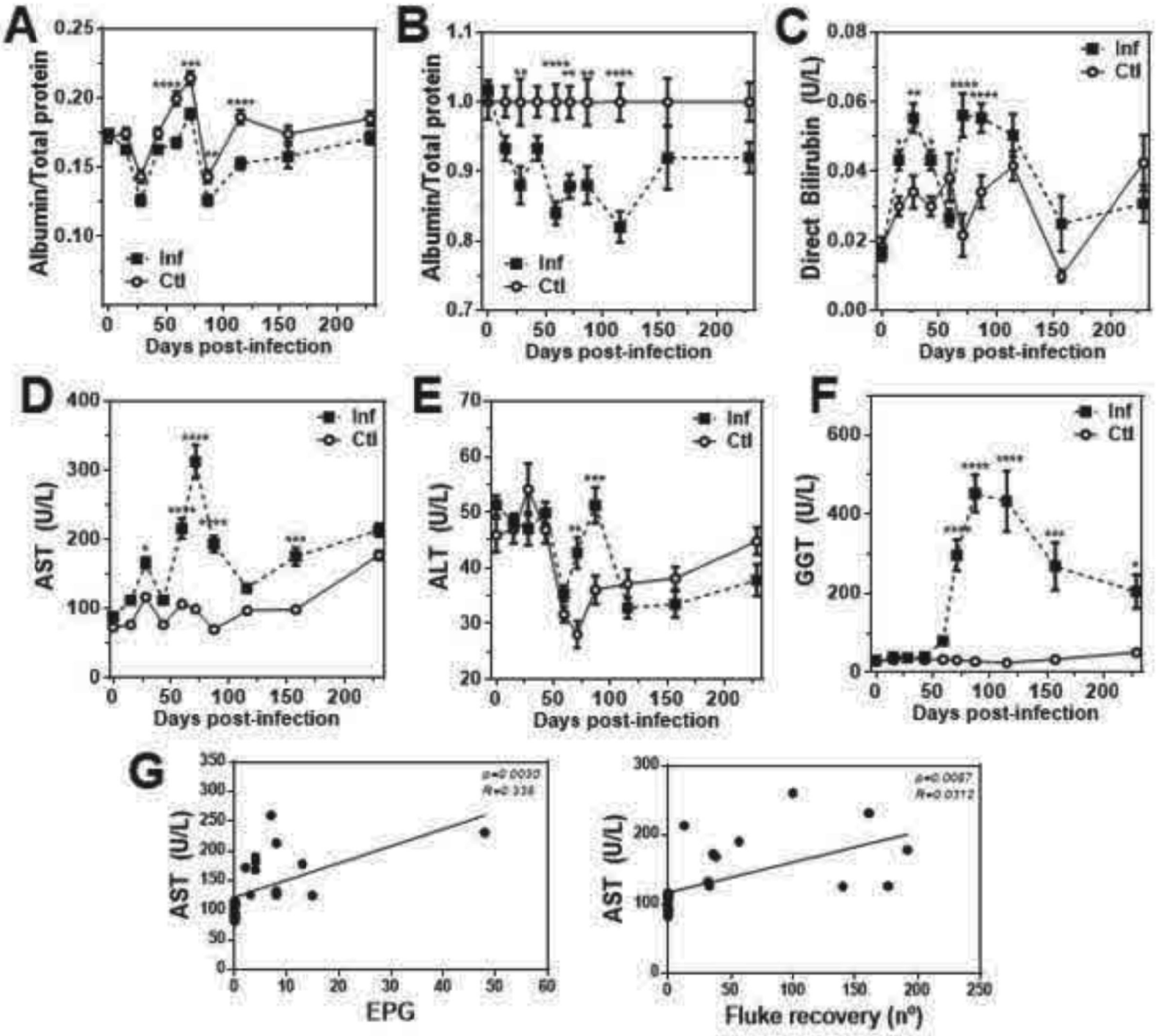
661

Figure 1




A
Eggs/gram (EPG)
Inf
Ctl
Days post-infection
B
Fluke recovery (n°)
Ctl
Inf
C
Fluke recovery (%)
Ctl
Inf
D
Fluke recovery (n°)
EPG
E
Fluke recovery (n°)
Liver Damage
F
G
H
I



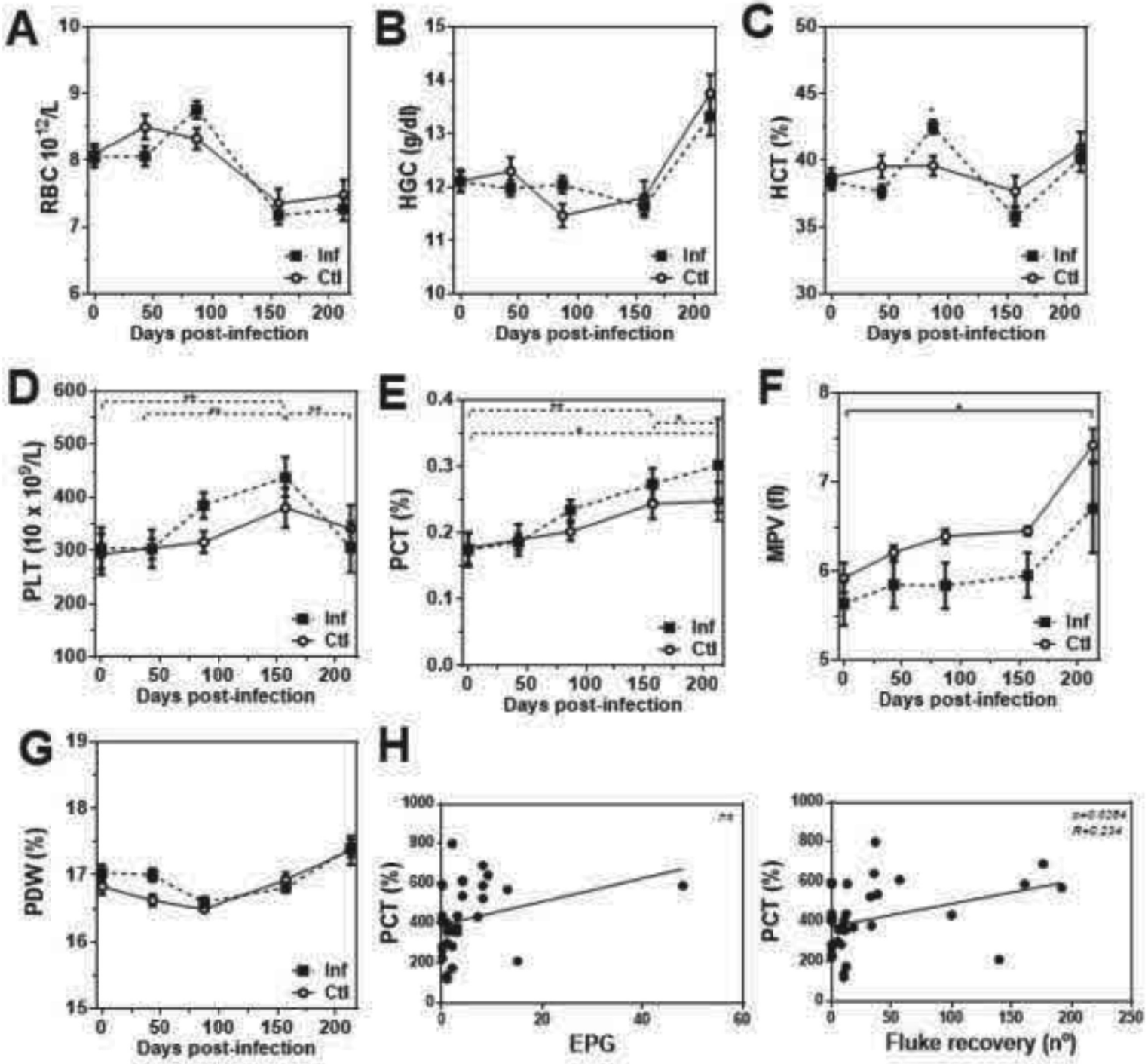


Figure 5

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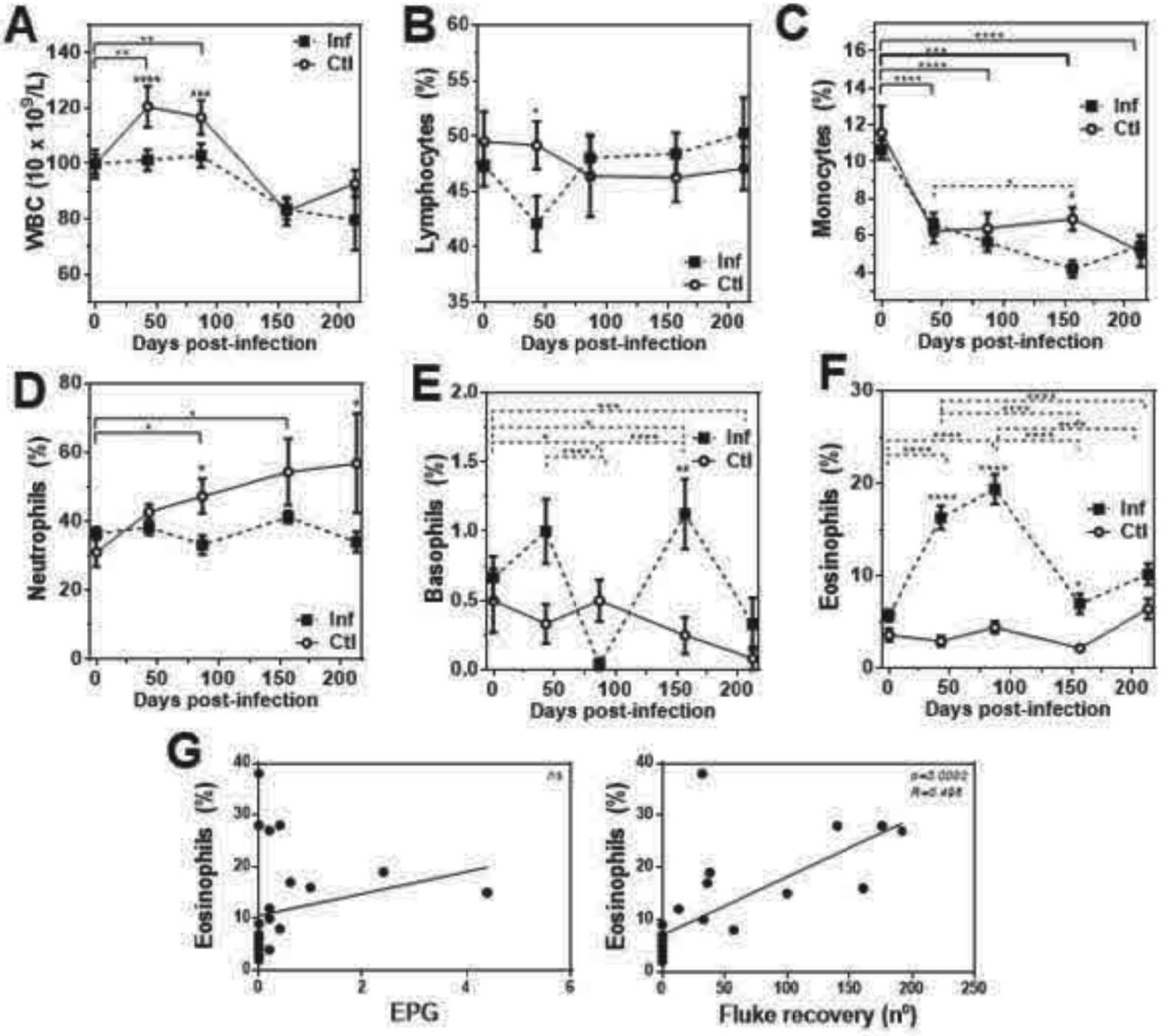

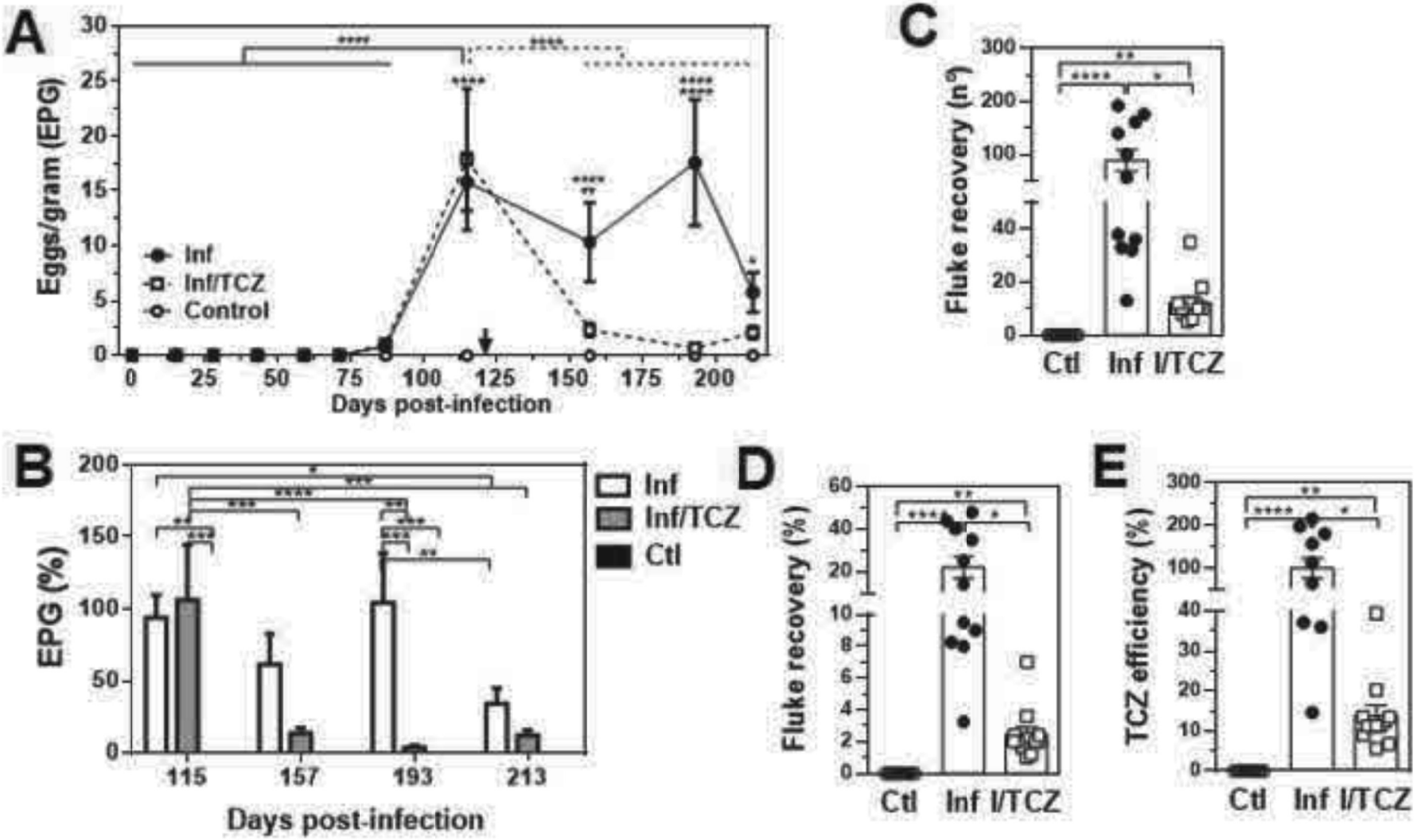

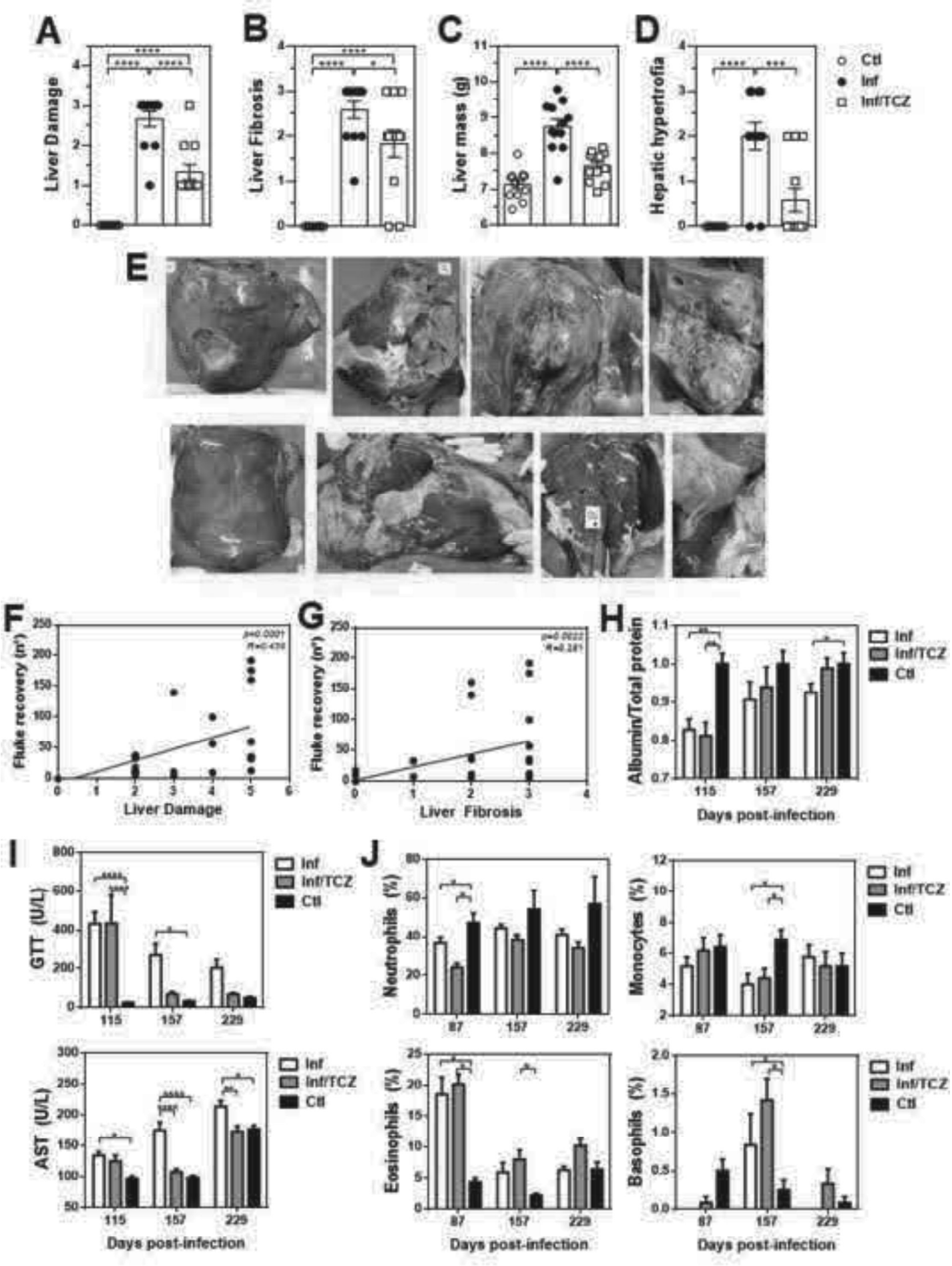
A
Liver Damage
B
Liver Fibrosis
C
Liver mass (g)
D
Hepatic hypertrofia
Ctl
Inf
Inf/TCZ
E
F
Fluke recovery (n°)
Liver Damage
G
Fluke recovery (n°)
Liver Fibrosis
H
Albumin/Total protein
Days post-infection
I
GTT (U/L)
AST (U/L)
Days post-infection
J
Neutrophils (%)
Monocytes (%)
Eosinophils (%)
Basophils (%)
Days post-infection

Title

Liver function markers and haematological dynamics during acute and chronic phases of experimental *Fasciola hepatica* infection in cattle treated with triclabendazole

Authors

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Credit author statement

Monique Costa: Investigation, data curation and analyses, original draft of the manuscript; Anderson Saravia: Biological sample collection and parasitology and reviewing the manuscript; Diego Ubios: Animal care and biological sample collection; Pablo Lores, Valeria da Costa, María Florencia Festari, Mercedes Landeira and Santiago A. Rodríguez-Zraquia: data curation and analyses and reviewing the manuscript; Georgget Banchero: Conceptualization and reviewing the manuscript. Teresa Freire: Conceptualization, experiment design and supervision, analyses of data and writing, review and editing the manuscript.