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Original Communication

Analysis of the humoral immune response (IgGa, IgGb and IgGc) in foals against *Gasterophilus* spp. (Diptera: Oestridae)

S. Miguélez[1], I. Francisco[1], A. M. Araújo[2], C. F. Cazapal-Monteiro[1], F. L. Arroyo[1], J. Sanchís[3], L. M. Madeira de Carvalho[4], R. Sánchez-Andrade[1], A. Paz-Silva[1] and M. S. Arias[1,*]

[1]Equine Diseases Study Group (Epidemiology, Parasitology and Zoonoses), Animal Pathology Department, Veterinary Faculty, Santiago de Compostela University, Campus Universitario, 27002-Lugo, Spain. [2]Universidade Lusófona, Lisboa, Portugal. [3]Parasitology, Faculty of Veterinary, University of La República (Regional Norte), Salto, Uruguay. [4]CIISA, Faculdade de Medicina Veterinária/Universidade de Lisboa, Pólo Universitário do Alto da Ajuda, Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal.

ABSTRACT

The dynamics of immunoglobulin G (IgG) subclasses (IgGa, IgGb and IgGc) were evaluated in foals infested by *Gasterophilus* spp., in an oceanic climate area (Northwest (NW) Spain). During a 1-yr period, blood samples were collected from sixteen 3-month-old foals, born in February, and their sera analyzed by using an enzyme-linked immunosorbent assay (ELISA) and excretory/secretory antigens from *G. intestinalis* second-stage larvae (GphiL2ES). With the purpose of assessing the possible effect of reinfestation on the humoral immune response, one group of eight foals was treated with moxidectin at the end of August. The antibody response increased during the summer, then decreased slowly till October and finally the highest values were observed in December; a marked reduction was observed in January and then the values decreased progressively till the end of the study (April). The kinetics of IgGa response registered higher values throughout the study, and the IgGb and IgGc recorded weak responses. Based on the high values that were recorded for the IgGa throughout the study, this response was analyzed to define the life-cycle of *G. intestinalis*. The increment in IgGa levels observed in June-July was related to the primary infestation of the foals; the higher values recorded between August and December, with the development of the active phase of the endogenous cycle; and the reduction from December to April, with a lessening in the numbers of L3s due to these instars exiting along with the feces. A similar response was recorded during reinfestation in the foals.

KEYWORDS: *Gasterophilus*, IgG subclasses, horses, ELISA

*Corresponding author: mariasol.arias@usc.es

INTRODUCTION

Gasterophilosis (Diptera: *Gasterophilus*) is a gastrointestinal myiasis that affects horses [1]. Adult flies of *Gasterophilus* deposit eggs on horses' hair, which are ingested by the horses and reach the mouth, where first instars (L1) hatch and migrate for several weeks, causing pain and inflammation, prior to their molt to L2s [2]. Second instar larvae attach to the buccal and pharyngeal mucosa for a brief period of time, and then move to the stomach or the intestine, where they molt to L3 instars. After a period of 5-8 months, L3s exit along with the feces and pupate in the soil. Because of the presence of maxillae, L2 and L3 instars attach to the non-glandular region (*G. intestinalis*), the gastric outlet and

the first part of duodenum (*G. nasalis*), causing small, shallow erosions which can evolve into inflammation and even rectal prolapse [3-6].

There is little information about the humoral immune response that horses develop against the *Gasterophilus* spp., and most of the investigations are centered on the analysis of the total IgG dynamics. Prior studies defined four IgG subclasses in horses (IgGa, IgGb, IgGc, and IgG (T)) [7]. This classification was later revised and IgGa was renamed as IgG1, IgGb as IgG4 + IgG7, IgGc as IgG6 and IgG (T) as IgG3 + IgG5 [8-10]. There is information available on the levels of IgG subclasses in infections by the equine influenza virus [11-13], *Streptococcus equi* [14-16], equine herpesvirus (EHV)-1 [17] and herpesvirus type 4 [18]. Nevertheless, there is a lack of information regarding their variations during the infestation by the parasites responsible for myiasis. The aim of the current work is to analyze the kinetics of IgG subclasses in foals from NW Spain, naturally infested by *Gasterophilus* spp.

MATERIALS AND METHODS

Geographical area of study

The current study was carried out in Northwest Spain (43°28'00" N, 07°37'19" W), an area with an oceanic climate [19] characterized by rainy winters and dry and slightly warm summers.

Indigenous Pura Raza Galega (PRG) foals were reared on a 50 Ha fenced area located in a mountainous region. These horses are valuable because they have an important role in limiting unwanted vegetal biomass, which reduces the risk of fire in forests and treed areas [20]. Their food sources are sparse and supplementation is seldom provided by their owners. Due to the difficulty to properly immobilize these horses, they were introduced into a chute before the collection of blood samples. Clinician veterinarians were responsible for the sampling and deworming in the study.

Experimental design

Between May 2014 and April 2015, blood samples were collected monthly from all the foals. Once obtained, an ELISA test was performed on the respective sera to detect the levels of IgG subclasses. Two groups of PRG foals were considered:

G-PI: Eight (two males and six females) 3-month-old foals maintained without treatment, throughout the study.

G-RI: Eight (two males and six females) 3-month-old foals that received 0.4 mg moxidectin/kg bodyweight (Cydectin, Fort Dodge, Spain) intramuscularly at the end of August 2014.

Antigen preparation

Larvae of *Gasterophilus* spp. were collected from the stomach and intestine of horses slaughtered at a local abattoir located in Galicia (NW Spain). The larvae were washed in phosphate-buffered saline (PBS, pH 7.4), and identified to species level [21, 22]. Accordingly, *G. intestinalis* second instars were separated to get their excretory/ secretory antigens (GphiL2ES), based on prior reports of the successful use of these antigens [23, 24]. For this purpose, L2s were incubated in Roswell Park Memorial Institute (RPMI) medium at 37 °C and 5% CO_2 atmosphere for 3 days, renewing the medium every 6-8 hours [24]. The medium was collected, dialyzed and lyophilized exhaustively against water. Prior to use, the protein concentration of GphiL2ES was estimated by using the bicinchoninic acid method (bicinchoninic acid (BCA) protein assay reagent; Pierce Biotechnology, Inc., Rockford, IL, USA).

ELISA protocol

The humoral immune response against *Gasterophilus* spp. was analyzed by means of an enzyme-linked immunosorbent assay (ELISA) and GphiL2ES. In brief, microtiter plates were coated with GphiL2ES at a concentration of 2.5 μg/mL in PBS and kept overnight at 4 °C; non-specific binding sites were blocked with PBS, 0.05% Tween and 1% skimmed milk (PTL), and serum samples were diluted to 1:100 in PTL. Horseradish peroxidase-conjugated goat anti-horse IgG (a, b or c) (Bethyl Laboratories®, USA) was used at a dilution of 1:1000 in PTL. Finally, a substrate consisting of 12 mg of *ortho*-phenylenediamine in 12 mL citrate buffer (pH 5.0) and 12 μL of 30% H_2O_2 was added.

The plates were incubated in the dark for 7 minutes at room temperature. The color reaction was stopped by the addition of 100 μL of 3 N H_2SO_4, and absorbances read using a spectrophotometer 680 XR; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 492 nm. In a previous study, specificity and sensitivity values of 89% and 78%, respectively, for this GphiL2ES-ELISA have been reported [24].

Sera pooled from twelve infested and eight uninfested horses were used as positive and negative controls, respectively. Positive-control sera were obtained from the horses that were found to have larval stages of *G. intestinalis* and *G. nasalis*, at the abattoir. Negative-control sera were collected from foals aged 2-3 months, which did not go outside and therefore had no possibility of being exposed to the bot fly.

Statistical analysis

The analysis of the values of IgGa, IgGb and IgGc by using the Kolmogorov-Smirnov test demonstrated that these humoral responses were not normally distributed (Statistic = 10.896, $P = 0.001$; statistic = 12.089, $P = 0.001$; statistic = 11.103, $P = 0.001$, respectively). Accordingly, these data were analyzed by means of the non-parametric Mann-Whitney *U* two-sided test (The significance level, also denoted as alpha or α, is the probability of rejecting the null hypothesis when it is true; $\alpha = 0.05$ indicates a 5% risk of concluding that a difference exists when there is no actual difference), and significant differences are considered when $P < 0.05$.

The existence of correlation between the values of IgG subclasses within the two groups of foals was assessed by means of the non-parametric Spearman's rank correlation test.

All tests were done using SPSS for Windows (V. 20.0; SPSS Inc., Chicago, IL, USA).

RESULTS

IgGa humoral response

As shown in fig. 1, the IgGa response against GphiL2ES in the G-PI increased from May and peaked in December. After this phase, the antibodies reduced strikingly till January, and then constant values were detected until the end of the study (April). Significant differences were observed (χ^2 = Chi-Square test of Independence; chi-squared distribution shows χ^2 on the x-axis and *P*-value on the y-axis; $\chi^2 = 50.460$, $P = 0.001$).

In the foals of G-RI, IgGa levels dropped after the administration of the macrocyclic lactone (Fig. 1). After lessening in October and November, the IgGa antibodies increased and the highest values were reached in December. From this month, levels reduced till January and then remained constant until the end of the assay ($\chi^2 = 42.900$, $P = 0.001$).

IgGb humoral response

In the foals of G-PI, the IgGb antibodies increased slightly from May to November (Fig. 2), and the highest levels were observed in December-January. Then the absorbances dropped steadily till March-April. These differences were significant ($\chi^2 = 44.247$, $P = 0.001$).

The kinetics of IgGb antibodies in the group G-RI decreased in October (1 month after the treatment with moxidectin) and increased again from November, reaching the highest values in December-February. The antibody levels lessened in March-April ($\chi^2 = 39.107$, $P = 0.001$).

IgGc humoral response

The IgGc values in G-PI increased gradually from June till December when the highest response was observed (Fig. 3). Then the absorbances decreased to reach the lowest values in April ($\chi^2 = 63.293$, $P = 0.001$).

In the foals of G-RI, minor variations were observed in the IgGc response throughout the assay (Fig. 3). Antibodies increased slightly till December, and then reduced till the end of the investigation (April), when the lowest values were obtained ($\chi^2 = 61.962$, $P = 0.001$).

Analysis of the IgG subclasses against *Gasterophilus intestinalis* excretory/secretory antigens

In the combined analysis of the three subclasses, the highest values were recorded for IgGa and the lowest for IgGc. Significant differences between the two groups of foals were obtained

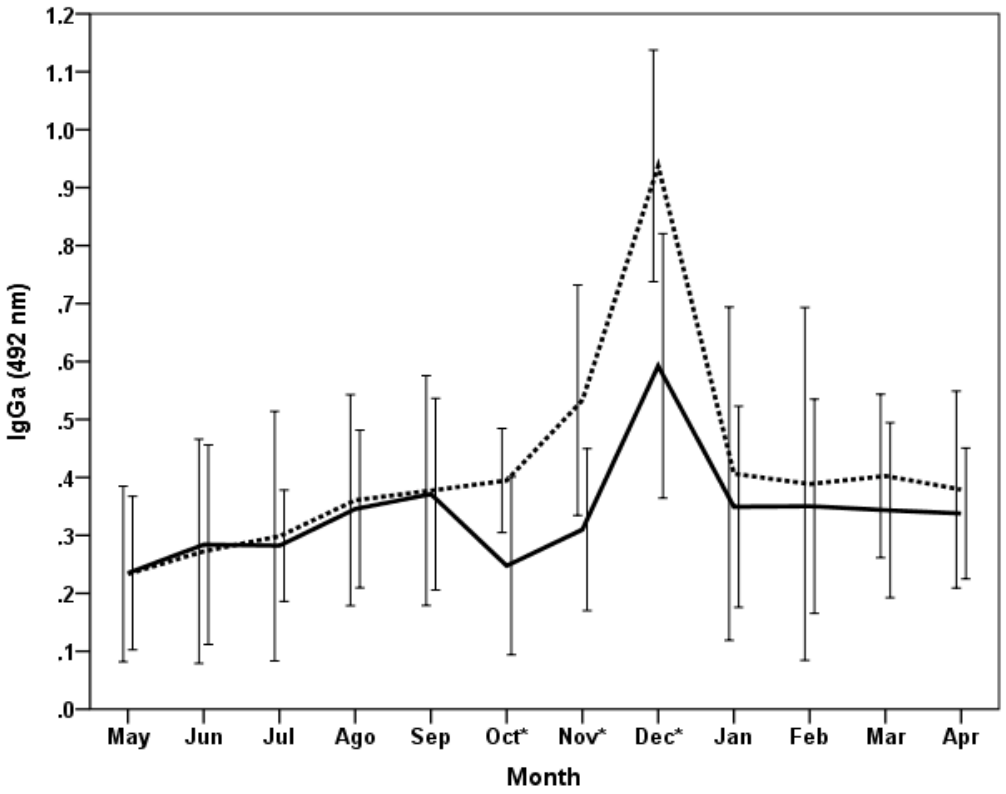

Fig. 1. Dynamics of IgGa antibodies against the excretory/secretory antigens from *G. intestinalis* L2 in Pura Raza Galega horses from NW Spain. G-PI, primary-infested foals (▪▪▪▪▪); G-RI, reinfested foals (——). *Significant differences between the two groups of foals. Peaks represent the average and vertical lines represent twice the standard deviation (2 * D).

for the IgGa (U = statistical value of the Mann-Whitney test; U = -2.972, $P = 0.003$), IgGb (U = -1.627, $P = 0.104$) and IgGc (U = -0.417, $P = 0.677$).

Similar patterns for the three subclasses were recorded in both groups, and statistical correlations were observed between the IgGa and IgGb values (Correlation coefficient $\rho = 0.381$, $P = 0.001$), IgGa and IgGc ($\rho = 0.247$, $P = 0.001$) and between IgGb and IgGc ($\rho = 0.293$, $P = 0.001$).

As shown in figs. 1 & 2, IgGa and IgGb antibody levels reduced after the administration of the macrocyclic lactone.

DISCUSSION

Horses become infested by the *Gasterophilus* spp. after the ingestion of eggs previously deposited by adult flies, in their hair, which can take place during spring, summer and autumn in oceanic climate areas [25]. With the aim of deepening the knowledge on the humoral immune response that horses develop against the *Gasterophilus* spp., serum variations in IgG subclasses (IgGa, IgGb and IgGc) were analyzed. Blood samples were collected from three-month-old foals and analyzed by means of an immunoenzymatic probe (ELISA) and the antigens from the second stage larvae of *G. intestinalis*. To ensure that exposure to *Gasterophilus* adult flies did not occur until then, foals born in February were used. At the beginning of the study, foals and adults showed a similar immune response. The highest values reached were for the IgGa subclass, and then for the IgGb, whereas only minor variations were observed for the IgGc response.

The levels of IgGa and IgGb increased from June, which seems to suggest that the primary infestation of the foals happened from April. In a previous investigation performed in the same area, the presence of adult flies was recorded

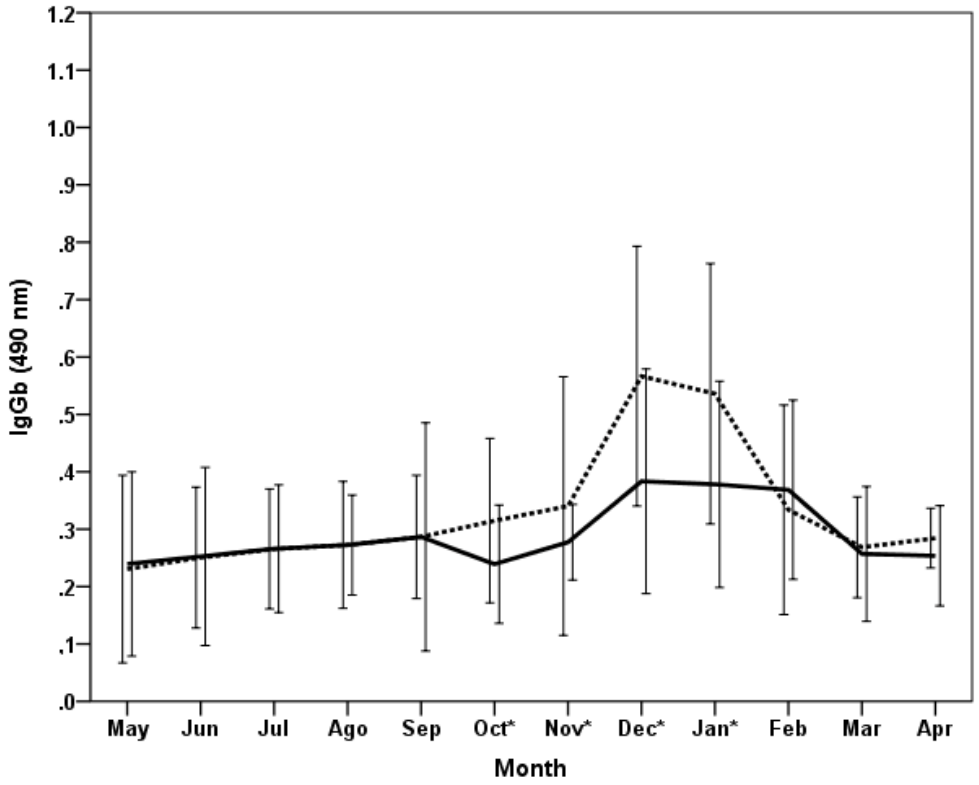

Fig. 2. Dynamics of IgGb antibodies against the excretory/secretory antigens from *G. intestinalis* L2 in Pura Raza Galega horses from NW Spain. G-PI, primary-infested foals (▪▪▪▪▪); G-RI, reinfested foals (——). *Significant differences between the two groups of foals. Peaks represent the average and vertical lines represent twice the standard deviation (2 * D).

from April-May [24]. Once the eggs have been deposited, a period of 8-10 days is needed for the development of the first instar (L1) larvae [26]. After the eggs containing the L1 are ingested, they hatch. The larval migration in the oral cavity differs according to the *Gasterophilus* species [27]. In the current investigation, the values of IgGa and IgGb rose steadily from July to October, reflecting the antigenic stimulus that took place during this period. It has been reported that the molting of L1 instars into second (L2) instars takes several weeks [2].

The second instar *Gasterophilus* larvae move to the esophagus and then to the stomach and intestine, where they molt into L3s, which remain affixed to the mucosa for 7-10 months, until winter ends [3]. The finding in the present investigation, of a significant increment in the IgGa and IgGb values between October and December could be attributable to the migration of the second instar larvae to reach their final destination and become L3 larvae. Previous works have demonstrated that L2s are more immunogenic than L3s, thus the reduction in the IgGa levels from December could be explained by taking into account the fact that the antigenic stimulus tends to decrease corresponding to the increase in L3s [28, 29]. Nevertheless, the delayed reduction in the IgGb response (from December to February) appears to point to a weak stimulus associated with L3s, possibly explained by the presence of hooked mouthparts and spines in these instars, responsible for damage consisting of hemorrhages, chronic gastritis, ulcers or even stomach rupture [25]. It should also be noted that in horses from areas with an oceanic climate, the presence of L3 instars reduces from March-April, when they drop onto the soil to pupate for a period of 2-6 weeks [24]. Then, horses maintained outdoors become frequently exposed to the flies emerging from the pupae, in the following summer.

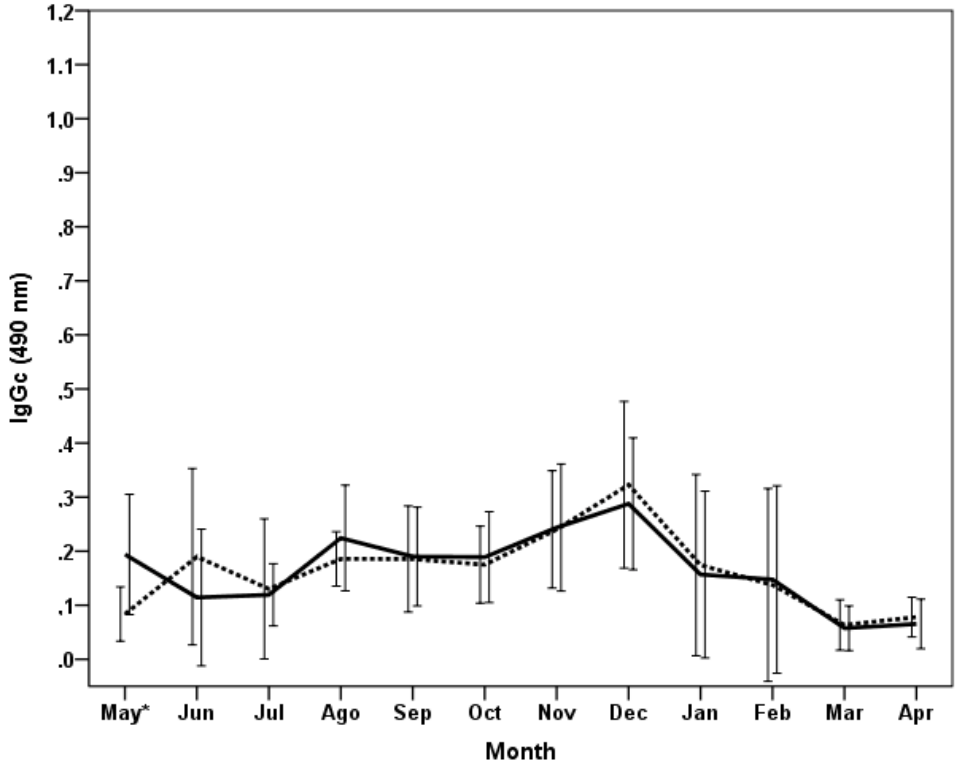

Fig. 3. Dynamics of IgGc antibodies against the excretory/secretory antigens from *G. intestinalis* L2 in Pura Raza Galega horses from NW Spain. G-PI, primary-infested foals (▪▪▪▪▪); G-RI, reinfested foals (——). *Significant differences between the two groups of foals. Peaks represent the average and vertical lines represent twice the standard deviation (2 * D).

In order to investigate the possible changes in the humoral immune response related to the reinfestation of horses by the *Gasterophilus* spp., one group of primary-infested foals was treated with moxidectin at the end of August. The purpose of this step was to allow the adult flies to lay eggs in the horses' hair. Moxidectin gel, administered orally to horses at 0.4 mg/kg body weight (BW), was 100 and 97.6% effective against third-instar *G. nasalis* and *G. intestinalis*, respectively [30]. However, moxidectin injectable is not licensed for use in equids. As occurred during primary infestation, significantly higher values were recorded for IgGa, and then for IgGb, but a small and almost constant IgGc response was detected throughout the assay. After dropping in October, the IgGa and IgGb responses rose quickly till December, and values lower that in the primary-infested, untreated foals were observed. While a reduction in IgGa values was achieved, IgGb levels remained constant between December and February. These data seem attributable to reinfestation during autumn. Thus the increment in IgGa (mainly) and IgGb could be related to the L1 hatching in the horse's mouth and the molt to L2 and then to L3. The delay in IgGb reduction could be correlated with the activity of the L3 larvae on the stomach and/or the intestine mucosa. Based on the presence of eggs on the horses' hair and the analysis of the L3 in the feces, and the dynamics of the total IgG in equines under oceanic climate conditions [25], the life-cycle of *Gasterophilus* spp. involves an egg-laying period from late spring and first instars in the mouth in early summer, L2s moving into the stomach and intestine during summer, L3 larvae remaining in this environment until the end of winter, pupation occurring in winter and adult bot flies emerging in spring.

There is a lack of data related to the humoral immune response (IgG subclasses) developed in horses infested by *Gasterophilus*. IgGa and IgGb

responses in the present investigation agree with those from a previous work conducted in the same geographical area (NW Spain), which found that the total IgG response among reinfested horses lessened between January and July and increased gradually from August to January, while in foals, total IgG response was detected from May [24].

CONCLUSION

Results collected during the present research suggest that the molting of L1 to L2 enhances the IgGa response both during the primary infestation and reinfestation of horses by the *Gasterophilus* spp., but that IgGb levels are lower and that the IgGc response is very weak. It can be concluded that the analysis of IgGa antibodies enables the detection of exposure of foals to horse bot fly. Besides, it is possible to correlate the IgGa humoral immune response with the active presence of L1 and L2 *Gasterophilus* larvae, in horses, which could be a highly useful method of diagnosis of equine infestation by the bot fly, and to establish the most suitable moment for deworming.

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CONFLICT OF INTEREST STATEMENT

No competing interests have been declared.

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