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EFFECT OF MALE ON THE AMINOPEPTIDASE ACTIVITY IN RABBIT SEMINAL PLASMA

TRABAJO FIN DE GRADO EN BIOTECNOLOGÍA

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EFFECT OF MALE ON THE AMINOPEPTIDASE ACTIVITY IN RABBIT SEMINAL PLASMA

Abstract

Since ovulation in rabbit is induced by sensory stimulation associated with mating, an effective control of ovulation is required in the artificial insemination process. The intramuscular application of GnRH analogues is the most common practice in rabbit reproductive management; however, the process can be simplified replacing the intramuscular application by the addition of the GnRH analogue to the semen extender, thus reducing the time needed to carry out the insemination, the risks associated with the use of needles and animal distress. The efficiency of GnRH analogue when administered with the semen extender depends on the degradation of the hormone by aminopeptidases, a group of enzymes present in seminal plasma. In this final degree project, aminopeptidase activity present in seminal plasma of males belonged to the New Zealand White line from five months (beginning of the training of those males used in artificial insemination) to nine months old (adult males with a normal semen production) is determined. For this, semen samples from each male were collected weekly and aminopeptidase activity was measured using three different substrates: alanine, cysteine and leucine-β- naphthylamide. Regarding aminopeptidase activity, it can be distinguished a group of males with a high aminopeptidase activity (around 1000 pmol/mg protein/min) and another group with half of the activity approximately. Correlation between quality sperm parameters and aminopeptidase activity was found, concretely with acrosome status and sperm abnormalities. Significant differences for aminopeptidase activity and male age or kinetic sperm parameters were not found. Regarding the results, it can be concluded that aminopeptidase activity varies among males and is not dependent on male age; this new parameter could be used to choose the best males for the artificial insemination procedure which involves GnRH analogue application with the semen extender.

Keywords: aminopeptidase activity, rabbit, artificial insemination, seminal plasma, semen.

Resumen

En la inseminación artificial del conejo, al ser una especie de ovulación inducida, se requiere un control efectivo de la ovulación, por lo que en el momento de la inseminación hay que inducir la ovulación, lo que habitualmente se hace mediante la aplicación intramuscular de un análogo sintético de la GnRH. El proceso puede simplificarse sustituyendo la aplicación intramuscular de la GnRH mediante su incorporación en el diluyente, lo que reduce el tiempo de aplicación de la dosis seminal a la vez que evita el riesgo asociado al uso de jeringuillas del personal que aplica la dosis y el estrés animal asociado al pinchazo. La eficacia de la hormona en el diluyente depende de varios factores, entre los que destaca la degradación del análogo por parte de las aminopeptidasas, unas enzimas presentes en el plasma seminal. En el presente Proyecto de Fin de Grado se determinó la actividad aminopeptidasa presente en el plasma seminal en machos de origen neozelandés desde los cinco meses de edad (inicio del entrenamiento de los machos utilizados en inseminación artificial) hasta los nueve meses de edad (machos adultos en plena producción de semen). Para ello, se recuperaron muestras de semen semanalmente de los machos y se determinó la actividad aminopeptidasa usando tres sustratos diferentes: alanina, cisteína y leucina-β-naftilamida. En base a los resultados obtenidos, se distingue un grupo de machos con alta actividad aminopeptidasa (alrededor de 1000 pmol/mg protein/min) y otro con una actividad de aproximadamente la mitad. Se observó cierta correlación entre algunos parámetros de la calidad del semen y la actividad aminopeptidasa, concretamente con el estado del acrosoma y anormalidades en el esperma. La edad de los machos no afectó significativamente a la actividad aminopeptidasa. Con los resultados obtenidos se puede concluir que la actividad aminopeptidasa varía en función del macho y es independiente de su edad, pudiéndose utilizar este nuevo parámetro para seleccionar los mejores machos para la técnica de inseminación artificial que utiliza GnRH en el diluyente.

Palabras clave: actividad aminopeptidasa, conejo, inseminación artificial, plasma seminal, semen.

Datos del Trabajo de Fin de Grado

Alumno/a**:** Ana Monserrat Martínez

Valencia, julio de 2014

D. José Salvador Vicente Antón

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En memoria a Ramonita, mi abuela, que habría sido quien más habría presumido de nieta graduada en el banquito donde se sentaba “a tomar la fresca” todas las tardes de verano.

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INDEX

FIGURE INDEX

TABLE INDEX

SYMBOLOGY AND ABBREVIATIONS

AI: artificial insemination.

ALH: amplitude of lateral head displacement about its average path

AR: acrosome reactivity.

BCA: bicinchoninic acid.

BNA: β-naphthylamide.

CASA: computer-assisted sperm analysis.

DTT: DL-dithiothreitol

LIN: linearity index (linearity of the curvilinear path).

LSD: least significant difference.

LSM: least square mean values.

MOT: percentage of total motile sperm.

NAR: normal apical ridge, percentage of sperm with intact acrosome.

PROG: percentage progressive spermatozoa.

SE: standard error.

STR: straightness coefficient (straightness of the path velocity).

VAP: average path velocity (velocity of the sperm head along its average).

VCL: curvilinear velocity (velocity of the sperm head along its actual curvilinear path).

VSL: straight line velocity (velocity of the sperm head along a straight line).

EFFECT OF MALE ON THE AMINOPEPTIDASE ACTIVITY IN RABBIT SEMINAL PLASMA

1. INTRODUCTION

Artificial insemination (AI) is the process of collecting sperm cells from a male and manually depositing them into the reproductive tract of a female. This reproductive technology permits an increase of male usage efficiency. In the AI process, semen is collected, diluted and extended to create hundreds of doses from a single ejaculate. This process supposes several advantages regarding traditional techniques: natural mating allows for the transfer of venereal diseases between males and females. Some pathogens can be also transmitted in semen through artificial insemination, but the collection process allows for the screening of disease agents. Collected semen is also routinely checked for quality, which can help to avoid problems associated with male infertility.

In AI, semen can be also easily transported, allowing multiple females in different geographical locations to be inseminated simultaneously, and stored for long periods of time, meaning that males can produce offspring long after their natural reproductive lives end. Additionally, artificial insemination increases the genetic selection potential because allows males to produce more offspring. Fewer males are needed, therefore, the best males can be chosen for use as parents, increasing the selection intensity. Furthermore, because males can have more offspring, their offspring can be used in a progeny test program to more accurately evaluate the genetic value of the male. Finally, individual farmers can use artificial insemination to increase the genetic pool with which the animals can be mated, potentially decreasing effects of inbreeding. The AI is the most important animal biotechnology applied to date on animal breeding.

The development of AI changed the rabbit industry, allowing more efficient management and reducing the production cost (Castellini, 1996). The main advantages were the reduction of the working time needed to reproduction tasks and the more efficient use of semen compared to natural breeding. The number of farms applying AI has progressively increased in recent years. It was estimated that overall 6.5 million doses of semen for AI were used per year in Spain (MINISTERIO DE AGRICULTURA, ALIMENTACIÓN Y MEDIO AMBIENTE, 2008).

However, it is necessary to take into account the particularities of each species in regard to AI. Ovulation in mammals is regulated by a complex neuroendocrine mechanism that involves signaling pathways between the reproductive organs and the brain, which involves pulsatile release of GnRH from the hypothalamus into the hypophyseal portal system with subsequent release of LH from the anterior pituitary into systemic circulation. Elevated circulating concentrations of LH induce a cascade of events within the mature follicle, culminating in ovulation (Figure 1). The broad classification of species as either spontaneous or induced ovulators is based on the type of stimulus responsible for eliciting GnRH release from the hypothalamus. In spontaneously ovulating species (e.g., human, sheep, cattle, horse, pigs), release of GnRH from the hypothalamus is triggered when systemic estradiol concentrations exceed a threshold. However, in rabbit, an induced ovulator, ovulation is not a regular cyclic event, and coitus is responsible for triggering GnRH secretion.

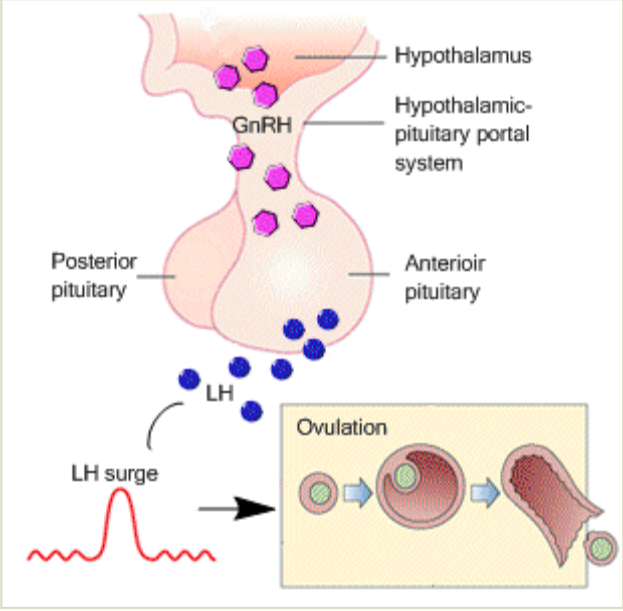

Figure 1. LH response to GnRH release from hypothalamus.

1.1 Artificial insemination in rabbit

Since ovulation in rabbit is induced by sensory stimulation associated with mating, an effective control of ovulation is required in the AI process. The first assays to control ovulation induction by GnRH analogues took place 25 years ago (Rodríguez and Ubilla, 1988; Theau-Clément *et al.*, 1990). The apparition of synthetic GnRH at the early 90s supposed one of the big hits in animal reproduction technologies. In rabbits, the GnRH analogue can also be used to eliminate cystic ovarian disease, to prevent embryo mortality, to synchronize the sexual cycle or even it has been demonstrated that the hormone can improve fertility rates in sheep, dairy cattle and buffalo, prolificacy rates in sheep and birth weight and perinatal survival rates in pig (Mawhinney *et al*., 1999; Peters *et al.*, 2000; Kaim *et al.*, 2003; Willard *et al*., 2003; Campanille *et al*., 2008).

Ovulation results obtained using GnRH analogues are similar to those obtained by natural mating (Theau-Clément *et al*., 1990). In addition, GnRH can be repeatedly injected without eliciting antibody formation (Adams, 1981).

In rabbits, AI is generally performed with 0.5 ml of fresh diluted semen which contains 4 to 20 million of viable spermatozoa (Viudes-de-Castro and Vicente, 1997; Castellini and Lattaioli, 1999; Viudes-de-Castro *et al*., 1999; Brun *et al.*, 2002) and females are induced to ovulate at the same time of insemination by means of an intramuscular injection of GnRH analogue.

Nowadays, the most widely used compounds are a natural decapeptide (gonadorelin) and a synthetic nonapeptide (buserelin) in aqueous solution, which is a structural analogue of GnRH and differs from the gonadorelin by the disposal of the terminal glycine and the replacement of glycine in position 6 by tert-butyl D-serine in the alcohol (Theau-Clément *et al*., 1990; Perrier *et al*., 2000).

To elicit a good LH response and to provide a good ovulatory response, an intramuscularly application of 0.8 µg buserelin is required (Foote and Simkin; 1993; Perrier *et al*., 2000).

The most frequent method used for ovulation induction in rabbit artificial insemination is intramuscular administration of GnRH analogue, but recently, several authors reported the possibility of ovulation induction by the addition of GnRH synthetic analogues to the semen extender applied when AI is performed (Quintela *et al.*, 2004; Viudes-de-Castro *et al.*, 2007; Ondruška *et al*., 2008; Vicente *et al*., 2011). This method supposes several advantages: less labour for the farmers, operating time and treatment distress for the operator and the animal. Elimination of needles from this type of practices caused a good reaction in the community, due to the depletion of the sanitary risk associated to their incorrect usage.

GnRH synthetic analogues can be absorbed via mucosa less efficiently than parenterally (about 5 times less); its absorption is influenced by the state of mucosa (Okada *et al*., 1984), mucosal peptidase activity (Acartürk *et al*., 2001), extender composition (organic acid level has positive effects on absorption) (Okada *et al*., 1982), formulation analogue (Padula, 2005) and by aminopeptidase activity of the seminal plasma (Viudes-de-Castro *et al*., 2014). Therefore, when GnRH analogue is added to the extender, 10 to 15 fold higher concentration must be applied to achieve similar fertility results than those obtained in the intramuscular administration (Viudes-de-Castro *et al.*, 2007; Zapletal and Pavlik, 2008).

1.2 Seminal vesicles

Seminal plasma consists of fluid produced in the *rete testis*, epididymis and the sex accessory glands. It is a complex medium containing a great variety of molecules. Several authors have found that the composition of seminal plasma varies among species, but also among males and among ejaculates from the same males (Maxwell and Johnson, 1999 and Pérez-Pe *et al*., 2001 in ram; Zhu *et al*., 2000 in boars; Viudes-de-

Castro *et al*., 2004 in rabbits). Components of seminal plasma play active roles in transportation and survival of viable spermatozoa in the female reproductive tract and in the elimination of non-viable spermatozoa from uterus (Troedsson *et al*., 2005).

Prostasomes are corpuscular structures with prostatic origin present in seminal plasma (Brody *et al*., 1983), which can be secreted by different cell types. They are membranous vesicles (150-200 nm diameter) containing a unique lipid composition (cholesterol, phospholipids as sphingomyelin, desmosterol), vitamin E, calcium and proteins (about 169 proteins has been detected by Utleg *et al*. (2003) in human prostasomes), some of them which are enzymes, and are involved in several physiological functions such as the enhancement of sperm motility (Stegmayr and Ronquist, 1982a), the liquefaction of semen (Lilja and Laurel, 1984) and immunosupresion (Kelly *et al*., 1991; Poiani, 2006). They also contain magnesium and zinc in concentrations that greatly exceed those of seminal plasma (Stegmayr and Ronquist, 1982a).

Appart from prostasomes, different vesicles can be found in seminal plasma as epidydimosomes and liposomes. Prostasomes, epididymosomes and liposomes are rising much interest about their role in reproductive performance, as it has been shown that pellet material containing prostasomes and amorphous substance favours progressive motility of washed spermatozoa (Stegmayr and Ronquist, 1982b); however, it was not elucidated whether prostasomes or amorphous substance or both contributed to the positive effect on progressive motility (Stegmayr and Ronquist, 1982a).

Rabbit semen contains different types of granules released by the anterior part of the prostate, representing about 15 to 20% of semen volume (Castellini *et al*., 2009). Granules protect the morphology of sperm, preserve the acrosome and the plasma membranes, so when they are not present, acrosome reaction (AR) is shown by sperm (Collodel *et al*., 2012).

In rabbit, there is a group of proteins in the seminal plasma that alters the GnRH analogue conformation, impeding its normal function in the female tract. This may be the reason why the concentration needed when the hormone is added together with the seminal dose is so elevated. This group of proteins belong to the aminopeptidase family.

1.3. Aminopeptidase activity

Aminopeptidase activity has been detected in seminal fluid, prostasomes and soluble membrane-bound spermatozoa fractions in man, but prostasomes showed the 65% of the total activity (Fernández *et al*., 2002). On the other hand, prostasomes have a key role in the modulation of flagellar motility (Fabiani *et al*., 1995), antioxidant and antibacterial properties (Saez *et al*., 2003; Burden *et al*., 2006) and are involved in the modulation of many other physiological parameters of ejaculated spermatozoa (Sullivan and Saez, 2013).

In rabbits, Vicente *et al.* (2011) showed how semen dilution rate affected the ovulation frequency when GnRH was added to the extender, concluding that the bigger the dilution, the better ovulation frequency was, and suggesting the increment of buserelin acetate availability due to the reduction of the aminopeptidases in the seminal dose as possible cause. In addition, it was tested that the dilution rate was not only important to diminish the quantity of seminal plasma but also the quantity of sperm present per dose: previous studies showed that pregnant rate of does was significantly affected by the seminal dose used and that there was a higher pregnancy rate in females inseminated with 6 million of spermatozoa rather than 24, 50 and 100 million, despite the total number of born and alive born were not significantly affected by the seminal dose (Vicente *et al.*, 2011). Therefore, the dilution rate of seminal plasma may determine the degradation rate of GnRH analogues and the concentration needed to provoke the ovulation induction.

Results from Viudes-de-Castro *et al.* (2014) showed an effect of rabbit genetic line on alanine aminopeptidase activity and observed higher aminopeptidase activity in

the paternal line (Line R) versus the maternal line (Line A), concluding that the differences found between research groups using the same synthetic analogue and bucks from different genetic lines could be due to differences in seminal plasma aminopeptidase concentration.

In general, the procedure of ovulation induction by the addition of GnRH synthetic analogues to the semen extender has been well accepted, but considering the social pressure in Europe for limiting or eliminating regular use of synthetic products in rearing it must be improved (Perrier *et al*., 2000). In addition, European Countries policy regarding meat residues and animal welfare and the exigency to maintain a "natural" image of rabbit meat is mediating the reduction in the use of hormones (Castellini, 1996).

2. OBJECTIVE

The main objective of this research is to study the effect of male and age of male (from 24 to 40 weeks) in the aminopeptidase activity and the possible effect of seminal characteristics on it.

3. MATERIAL AND METHODS

Unless stated otherwise, all chemicals in this study were purchased from Sigma-Aldrich Química S.A (Madrid, Spain).

3.1 Animals

All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013; BOE = Official Spanish State Gazette).

To study the effect of male and age on aminopeptidase activity, a total of 18 adult bucks belonging to a maternal rabbit line (Line A) were used. Line A (New Zealand White line) has been selected since 1980 for litter size at weaning with a family index (Estany *et al*., 1989) (Figure 2).

Figure 2. New Zealand White line rabbit.

3.2 Environmental conditions and animal management

All males were kept individually in flat deck cages under 16 h light / 8 h dark conditions at the experimental farm of the Animal Technology and Research Centre (CITA-IVIA, Segorbe, Castellón, Spain) and fed *ad libitum* with the same commercial diet (17.5% crude protein, 2.3% ether extract, 16.8% crude fibre, 2.600 kcal DE/Kg) and had free access to water.

3.3 Semen collection and evaluation

All males began their training to use an artificial vagina (Figure 3) at five months. One ejaculate was collected per male each week for the first two weeks. After this period, two ejaculates per male were collected once a week, with a minimum period of 30 min between ejaculates. Only ejaculates containing urine or with a volume equal or less than 0.1 ml were rejected. Ejaculates coming from the same male and the same day were pooled together. After collection, sperm evaluation was performed to assess the initial seminal quality.

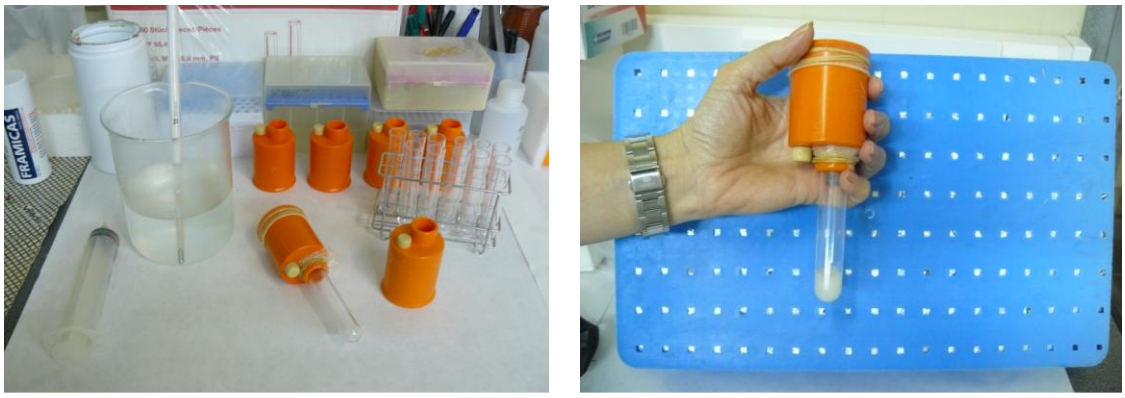

Figure 3. Semen collection material and ejaculate obtained using an artificial vagina.

One 10 µL aliquot from each ejaculate was diluted 1:10 with Tris-citrate-glucose extender (250 mM tris-hydroxymethylaminomethane, 83 mM citric acid, 50 mM glucose, pH 6.8-7.0, 300 mOsm·kg^{-1}) for a motility rate evaluation and a second 20 µL aliquot was fixed with 180 µL of a solution of glutaraldehyde 2% in phosphate buffered saline to calculate the concentration, the sperm abnormalities and the status of the acrosome (percentage of sperm with intact acrosome, NAR).

Sperm motility was assessed at 37ºC at 10X using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer, C.C.A, Microptic, Barcelona, Spain). The following kinetic parameters were evaluated: percentage of total motile sperm (MOT, number of motile sperms/total x 100); average path velocity (VAP, velocity of the sperm head along its average; µm/s); curvilinear velocity (VCL , velocity of the sperm head along its actual curvilinear path; µm/s); straight line velocity (VSL, velocity of the sperm head along a straight line; µm/s); linearity index (LIN, linearity of the curvilinear path, average value of the ratio VSL/VCL, %); straightness coefficient (STR, straightness of the path velocity VSL/VAP, %); percentage progressive spermatozoa (PROG, percentage of spermatozoa with a VAP>40 µm/s and straightness >80%) and amplitude of lateral head displacement about its average path (ALH; µm) (Lavara *et al.*, 2008).

Concentration was evaluated using a Thoma chamber. Percentage of normal apical ridge was observed by phase contrast at a magnification of 1200X.

3.4 Seminal plasma preparation

Semen samples were centrifuged at 10,000 g for 10 min at 22 ºC. The resulting supernatants were collected and centrifuged again (10,000 g for 10 min) to remove residual spermatozoa and cell debris. The resulting pellets were discarded, while the supernatants were put into a different tube, labeled properly and stored at -80ºC until use (Figure 4).

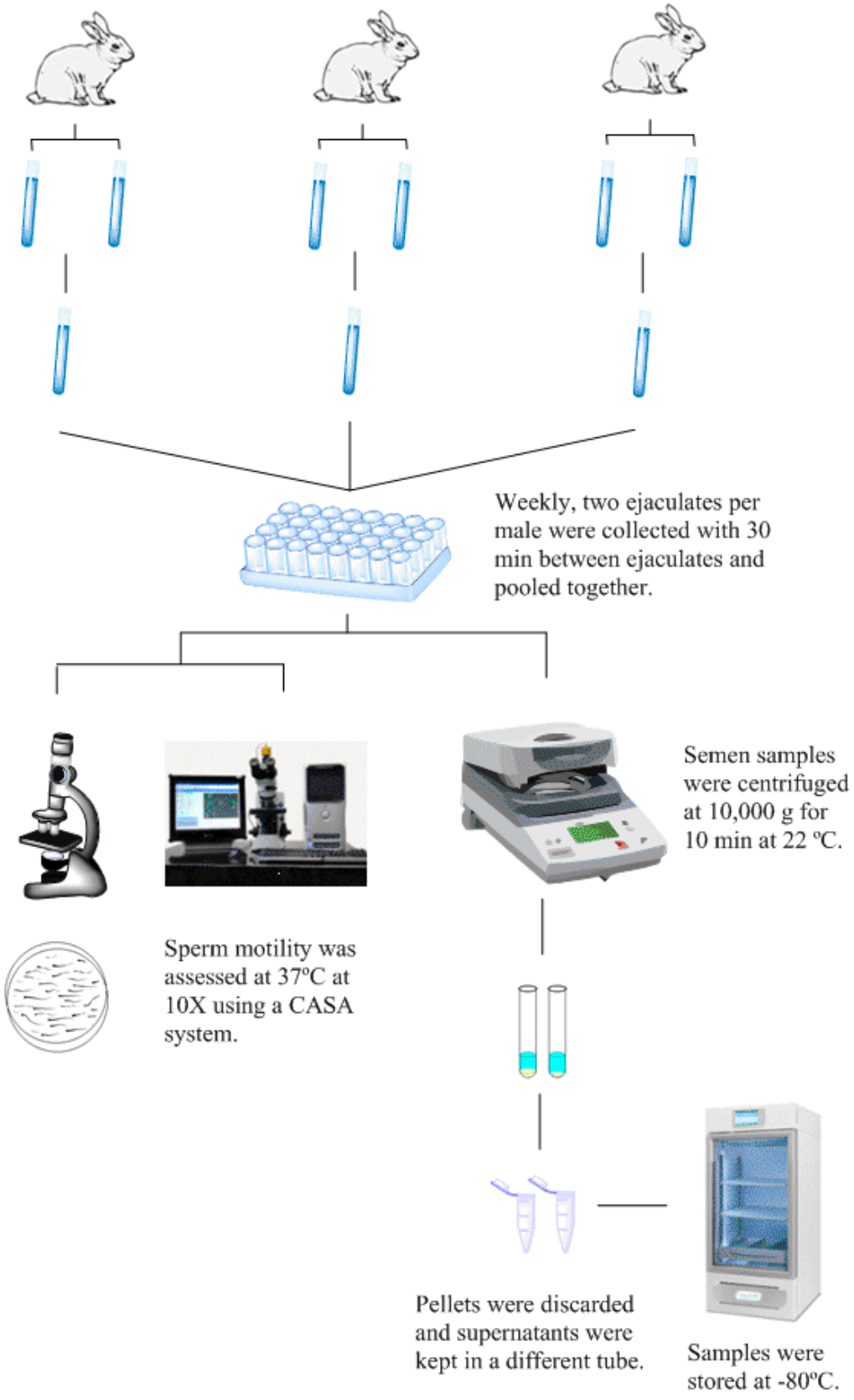

Figure 4. Scheme of the experimental methodology used to obtain seminal plasma.

3.5 Determination of aminopeptidase activity.

3.5.1 Measurement of protein concentration.

Protein concentration was measured using the bicinchoninic acid assay (BCA) (Thermo Scientific™ Pierce™ BCA Protein Assay Kit) using bovine serum albumin as the standard (Smith *et al.*, 1985). This assay is used for colorimetric detection and quantification of total protein (Figure 5).

The principle of the BCA assay relies on the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by the reduction of Cu^{2+} to Cu^{1+} in a concentration-dependent manner. Bicinchoninic acid is a highly specific chromogenic reagent for Cu^{1+}, forming a purple complex by the chelation of two molecules of BCA with one cuprous ion, with a maximum absorbance at 562 nm.

Figure 5. Material used for the measurement of protein concentration.

The assay was performed in a 96-well transparent polystyrene microplate and protein concentration and standards were measured in duplicates (Figure 6).

The BCA working reagent (200 µL) was added to the samples, mixed thoroughly on a plate shaker for 30 seconds and the microplate was incubated at 37ºC during 30 minutes. The microplate was allowed to cool to room temperature afterwards and absorbance of the solution at 562 nm was measured in a plate reader. The average 562 nm absorbance measurement of the blank standard replicates was subtracted from the 562 nm measurements of unknown sample replicates.

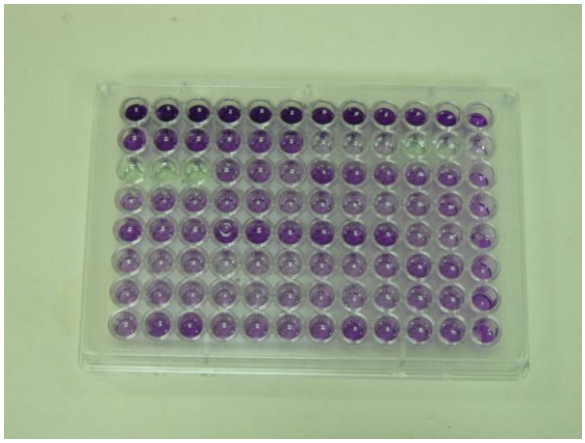

Figure 6. Protein concentration assay in a 96-well transparent polystyrene microplate.

In order to obtain the values of protein concentration of each sample, a standard curve was prepared by plotting the average blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/mL.

3.5.2 Measurement of aminopeptidase activity

Aminopeptidase activity was fluorometrically measured by a modification of the Greenberg method (Greenberg, 1962) using alanine-β-naphthylamide, leucine-β-naphthylamide and cysteine-β-naphthylamide as substrates (Figure 7).

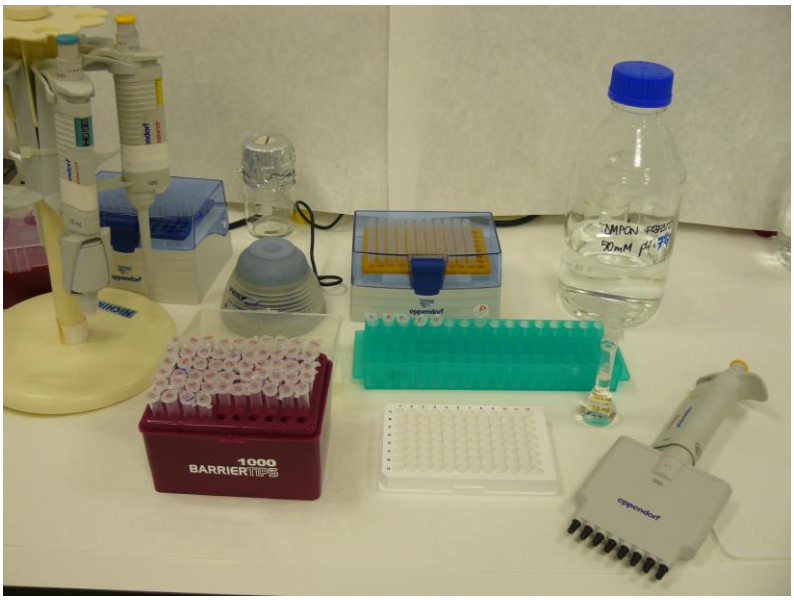

Figure 7. Material used for the measurement of aminopeptidase activity.

The assay is based on the fluorescence of β-naphthylamide (BNA) released from the hydrolysis of an appropriate substrate by the enzyme. This release is the result of the incubation in a 96-well white polystyrene microplate of 15 µL of seminal plasma sample with 100 µL of substrate solution. The components of the substrate solutions (in a total volume of 100 µL) included 50 mM sodium phosphate buffer (pH 7.4), 0.01 mg of BSA, 2 mM DL-Dithiothreitol (DTT) and the appropriate substrate used in each case (0.125 mM alanine-β-naphthylamide, 0.125 mM leucine-β-naphthylamide and 0.063 mM cysteine- β-naphthylamide, respectively). After incubation for 30 min at 37ºC, 100 µL of 0.1 M sodium acetate buffer (pH 4.2) was added to the mixture to terminate the reaction. The released BNA was determined by measuring the fluorescence intensity at 460 nm with excitation at 355 nm (Figure 8 and 9). Wells without sample were used to determine the background fluorescence. The relative fluorescence was converted to pmol of released β-naphthylamide by comparison with a standard curve, previously obtained with increasing concentrations of product and decreasing concentrations of substrate. Enzyme activity was measured in triplicate.

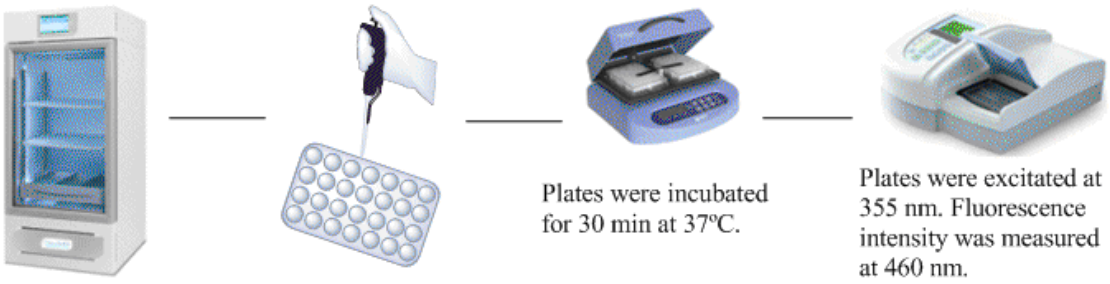

Figure 8. Scheme of the experimental methodology used to analyze aminopeptidase activity.

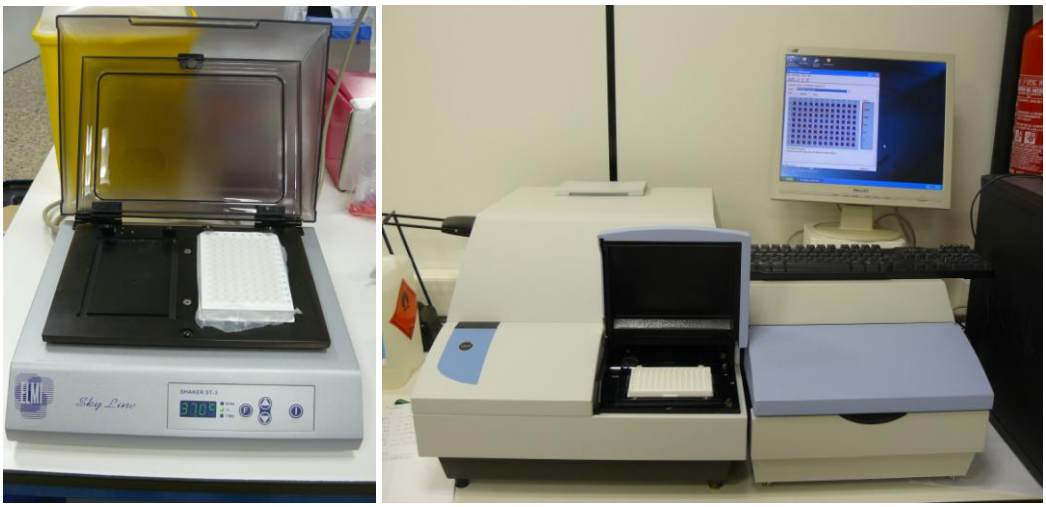

Figure 9. Incubation during aminopeptidase activity measurement and plate reader.

The peptidase activity was expressed as pmol of β-naphthylamide released per mg of protein per min by comparison with a correspondent standard curve. The existence of a linear relation between time of hydrolysis and protein content in the fluorometric assay was a previous condition.

3.6 Statistical analysis

Data were statistically evaluated with Statgraphics® Plus 5.1 library procedures (Statistical Graphics Corp., Rockville, MO, USA). To analyze the effect of male (1 to 18) and age of male (24 to 40 weeks) on aminopeptidase activity, a two way analysis of variance (ANOVA) was used. Further, in the aminopeptidase activity analysis, a

covariance analysis including semen production or percentage of normal apical ridge as covariate had initially been included in the model. Means were separated using the Fishers Least Significant Difference (LSD) test at a fixed 5% error level and the results are presented as the least square mean values (LSM) ± the standard error (SE). Multiple variable analysis procedure was used to calculate the correlations between pairs of seminal parameters studied using the Pearson product-moment correlation coefficient.

4. RESULTS

Aminopeptidase activity was detected in all seminal plasma samples. As shown in Figure 10 the activity evolution develops the same pattern regardless the substrate used. It is apparent that the highest level of activity was found for leucine and alanine peptidases and a moderate activity was found for cysteine peptidase.

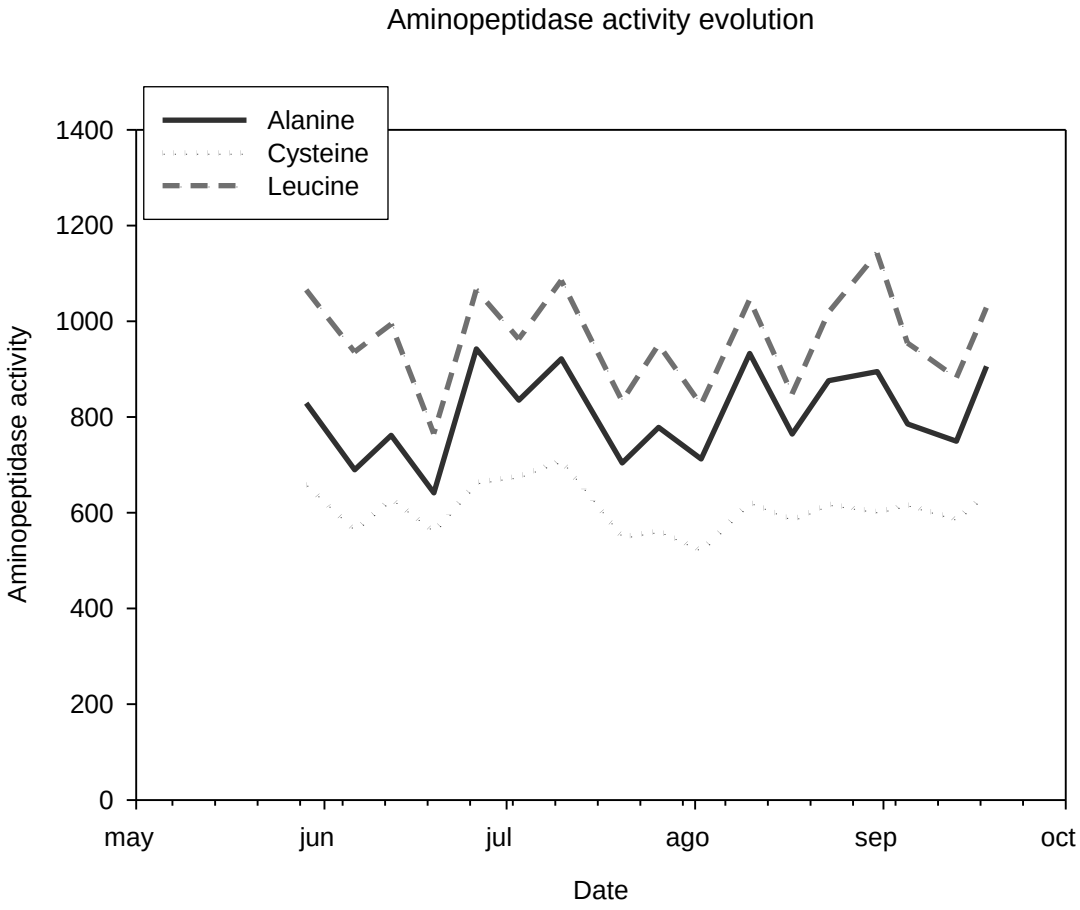

Figure 10. Aminopeptidase activity along the experimental period studied (pmol of β-naphthylamide released per mg of protein per min).

No significant difference was found for age of male on the aminopeptidase activity. Results presented in Table 1 indicated that aminopeptidase activity was different between males ($P<0.05$). The covariates used (semen production or percentage of normal apical ridge) were no significant.

Table 1. Aminopeptidase activity in seminal plasma (LSM ± SE).

Male	Nº of ejaculates	Alanine aminopeptidase activity (pmol/mg protein/min)	Cysteine aminopeptidase activity (pmol/mg protein/min)	Leucine aminopeptidase activity (pmol/mg protein/min)
1	10	375.3 ± 86.4 [a]	323.0 ± 64.7 [a]	556.1 ± 98.9 [a]
2	12	446.6 ± 79.1 [ab]	387.1 ± 59.3 [ab]	715.9 ± 90.6 [abc]
3	11	452.9 ± 82.6 [abc]	379.2 ± 61.9 [ab]	577.1 ± 94.6 [ab]
4	11	574.1 ± 82.4 [abc]	518.3 ± 61.8 [bcd]	591.7 ± 94.4 [ab]
5	8	586.9 ± 97.3 [abcd]	578.5 ± 72.9 [cde]	674.3 ± 111.4 [abc]
6	14	639.0 ± 73.2 [bcd]	528.5 ± 54.8 [bcd]	813.4 ± 83.8 [bcd]
7	9	691.4 ± 91.4 [cde]	566.0 ± 68.5 [cd]	914.5 ± 104.7 [cd]
8	15	798.4 ± 70.6 [def]	585.7 ± 52.9 [f]	1027.3 ± 80.9 [d]
9	12	852.7 ± 78.9 [efg]	750.2 ± 62.0 [ef]	862.6 ± 90.4 [cd]
10	13	884.0 ± 76.0 [efg]	576.9 ± 56.9 [cd]	996.2 ± 87.0 [d]
11	13	944.4 ± 75.8 [fgh]	458.0 ± 56.8 [abc]	1019.9 ± 86.8 [d]
12	7	989.7 ± 104.4 [fgh]	720.3 ± 84.9 [def]	1026.7 ± 119.5 [de]
13	12	1005.0 ± 79.6 [fgh]	648.8 ± 59.7 [de]	1345.9 ± 91.2 [f]
14	14	1038.3 ± 73.2 [gh]	629.3 ± 54.8 [de]	963.6 ± 83.8 [d]
15	14	1039.9 ± 73.2 [gh]	622.1 ± 54.8 [de]	1304.8 ± 83.8 [ef]
16	7	1075.8 ± 105.1 [gh]	909.3 ± 84.8 [f]	1026.8 ± 120.4 [de]
17	9	1090.3 ± 92.1 [gh]	836.3 ± 69.1 [f]	1330.2 ± 105.5 [ef]
18	11	1139.5 ± 82.4 [h]	856.0 ± 61.7 [f]	1459.6 ± 94.4 [f]

[a,b,c,d,e,f,,g,h] : Values within a column with different superscripts differ significantly at $P < 0.05$.

As shows Figure 11, two groups of males can be can be clearly distinguished: males with high or low activity. What is interesting in this data is that aminopeptidase activity of one group is almost twice than the other group and that approximately half of population can be configured in each group.

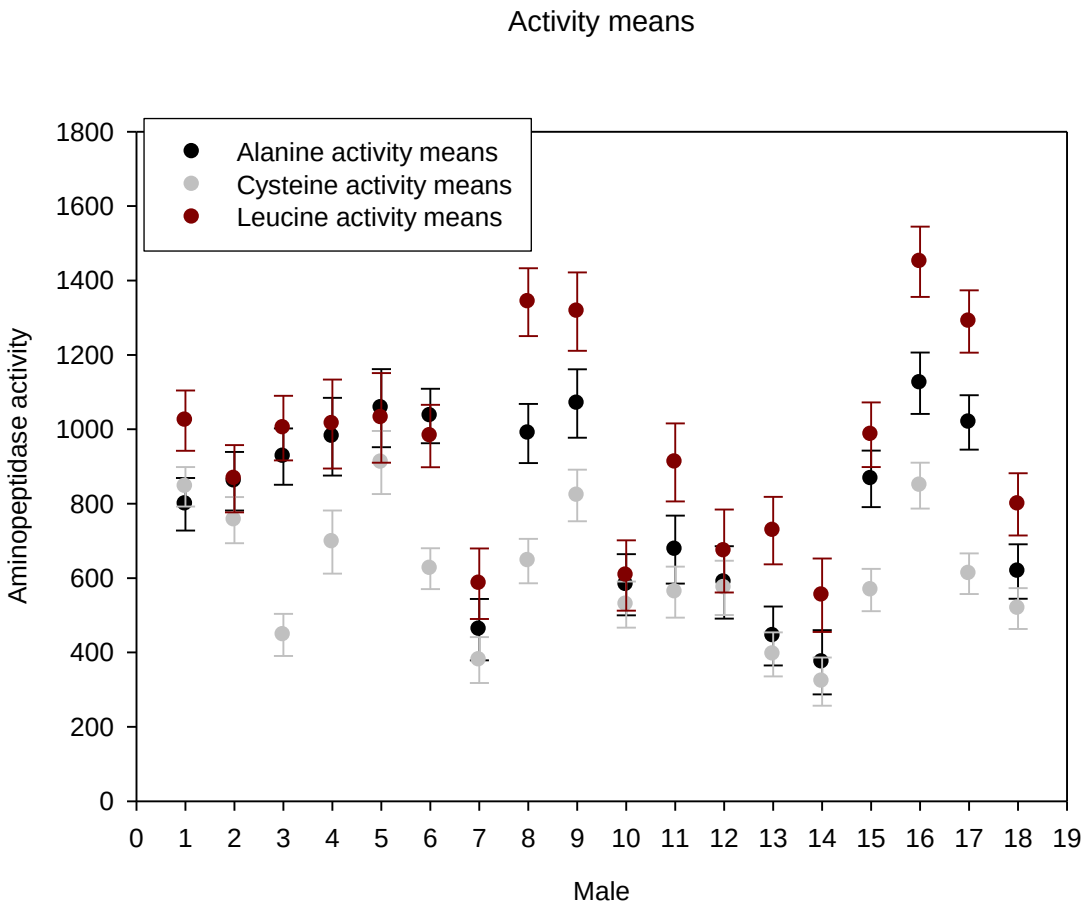

Figure 11. Mean aminopeptidase activity per male and substrate (pmol of β-naphthylamide released per mg of protein per min).

The results for the study of correlation between quality sperm characteristics and aminopeptidase activity are showed in Table 2. Kinetic parameters were no correlated with the aminopeptidase activity. With respect to the quality parameters, some of the correlations were significant. There was a significant negative correlation between the status of the acrosome and the alanine aminopeptidase activity as well as leucine aminopeptidase activity. Then aminopeptidase activity could be related with sperm damaged acrosome. In the case of the sperm abnormalities, it showed a significant positive correlation with alanine and leucine aminopeptidase activity.

Table 2. Pearson's correlation coefficients between sperm traits and aminopeptidase activity.

	Alanine aminopeptidase activity	Cysteine aminopeptidase activity	Leucine aminopeptidase activity
CONC	-0.0088	-0.0117	-0.0659
NAR	-0.2554**	0.1184	-0.2171**
ABNOR	0.2323**	0.0469	0.2398**
MOT	-0.1103	0.0409	-0.0544
VAP	-0.0622	0.0213	0.0463
PROG	-0.0711	-0.0172	-0.0033
VCL	-0.0998	0.0240	0.0077
VSL	-0.0314	0.0208	0.0743
LIN	0.0245	-0.0528	0.0456
STR	0.0399	-0.0188	0.0541
ALH	-0.1087	-0.1183	-0.0397

CONC: Sperm concentration per mL; NAR: % of normal apical ridge; ABNOR: % of sperm abnormalities; MOT: % of total motile sperm cells; VAP, average path velocity (μm/s); VCL: velocity of the sperm head along its actual curvilinear path (μm/s); VSL: straight line velocity (μm/s); LIN: linearity index (%); STR: straightness coefficient (%); PROG: percentage of progressive spermatozoa, ALH: amplitude of lateral head displacement (μm). **: Significant effect at $P < 0.001$.

5. DISCUSSION

The present study clearly demonstrates that aminopeptidase activity varies between males and that it results characteristic for each buck independently of its age.

In rabbit artificial insemination, ovulation induction requires the use of exogenous hormone. The intramuscular administration of synthetic GnRH analogues is the most common practice in the industrial rabbit production. However, there is one method of inducing ovulation intravaginally where the GnRH analogue is incorporated into the extender. In this method, GnRH analogues are susceptible to peptidase degradation. The effectiveness of the intravaginally administration depends on the degradation of the analogue by the enzymes present in the seminal plasma, among others. As consequence, when GnRH analogue is incorporated into the extender, it is necessary to increase its concentration considerably to induce ovulation.

Aminopeptidase activity has previously been reported in seminal plasma of different species of mammals (Agrawal and Vanha-Perttula, 1986; Huang *et al*., 1997; Osada *et al*., 2001; Fernández *et al*., 2002; Irazusta *et al*., 2004; Viudes-de-Castro *et al*., 2014).

The absence of large variations over time in the aminopeptidase activity in semen observed in the present work offers the possibility of establishing a new solution to the problem previously mentioned: the selection of those males with a low aminopeptidase activity for AI would allow the use of lower hormone concentrations in the extender.

The absorption of GnRH by the vaginal mucosa is influenced by the extender composition, the state of the mucosa, and by aminopeptidases present in the seminal plasma (Okada *et al*., 1982 and 1984; Viudes-de-Castro *et al*., 2014). Improving the

absorption of the GnRH analogue in the vagina could be one of the possible solutions. A good definition of how parameters affect the absorption process and the determination of the best conditions to carry out this method, could improve vaginal absorption and thus, the concentration of hormone needed would diminish.

On the other hand, the use of those GnRH analogues with a higher resistance to peptidase activity could also ameliorate this method, as increasing analogue resistance to aminopeptidases would imply that using less hormone dose the same effect could be achieved. Also studies about how to increase the analogue life span could be performed, as for example by the blockage of determined enzyme cleavage sites of the protein or the addition of chemical groups coating the analogue, so it would be protected. These options would affect GnRH analogue availability in the female tract.

The co-administration of certain peptidase inhibitors with the seminal dose could also guarantee the reduction of the GnRH analogue needed. Ovulation rate in females should be tested to observe the proper behavior of the inhibitors. However, aminopeptidase inhibitors as phenanthroline, EDTA or bestatine could result toxic to spermatozoa because they can alter spermatozoa membrane so they would not be capable to fecundate the oocyte. This is, despite aminopeptidase activity was diminished and hormone stability improved, the dose would not be useful for AI.

It is well known that some of the factors that influence the fertility and prolificacy results after AI are the sperm abnormalities and the acrosome status. The present work also shows that aminopeptidase activity is related with those factors: the better the integrity of the acrosome and lower the percentage of abnormal forms, the lower the aminopeptidase activity. Several studies in different species corroborate the relationship between abnormal sperm rate and fertility reduction (Xu *et al*., 1998; Farrel *et al*., 1993; Lavara *et al*., 2005).

Considering the fact that some semen quality traits are related with the aminopeptidase activity and it does not vary with age, the determination of aminopeptidase activity may be included as a new parameter in the basic analysis of semen during the male training period. This new parameter would provide information about which males are more likely to be used when GnRH analogue is added to the semen extender (those with the lowest aminopeptidase activity) and maybe, if this trait shows a high heritability, it is possible the future creation of male lines not only regarding productivity but also aminopeptidase activity. Several studies should be done in the different lines used traditionally for AI, as male genotype plays a key role in semen characteristics (Theau-Clément, 2003).

Several of the above mentioned strategies could be carried out jointly, which could reduce the amount of GnRH analogue to be added to the extender to trigger ovulation similar to its intramuscularly application levels, and additionally, the risks associated with the use of needles would be eliminated from rabbit AI.

On the other hand, new studies to define annual variability of aminopeptidase activity of males must be performed. Several authors have found that the composition of seminal plasma varies among species, among males, and among ejaculates from the same male (Maxwell and Johnson, 1999; Pérez-Pe *et al*., 2001 in rams; and Zhu *et al*., 2000 in boars) (Annex 1). The collection of samples in this experiment ended in September, so possible changes in aminopeptidase activity throughout the different seasons can not be observed, and since the effect of high summer temperatures in semen does not appear until November-December due to the duration of spermatogenesis, more research should be done on the possible seasonal variation of aminopeptidase activity.

6. CONCLUSION

This study demonstrates that aminopeptidase activity of seminal plasma varies among males from the same line in rabbits. This fact opens up new prospects for improving the intravaginal administration of GnRH analogues method, as males with lower activity could be chosen and employed in this technique. This will allow the reduction of the hormone needed for the process, which is one of the major goals in the intravaginal GnRH application method nowadays. Thus, the technique becomes a safer, welfare-oriented and more affordable technique compared with the traditional ones.

7. BIBLIOGRAPHY

ACARTÜRK, F.; PARLATAN, Z.; SARACOGOLU, F. (2001). Comparison of vaginal aminopeptidase enzymatic activities in various animals and in humans. *Journal of Pharmacy and Pharmacology*, 53:1499-1504.

ADAMS, C.E. (1981). Artificial insemination in the rabbit: The technique and application to practice. *Journal of Applied Rabbit Research,* 4: 10-13

AGRAWAL, Y.; VANHA-PERTTULA, T. (1986). Alanyl aminopeptidase of bovine seminal vesicle secretion. *International Journal of Biochemistry*, 18 (8): 725-9.

BRODY, I.; RONQUIST, G.; GOTTFRIES, A. (1983). Ultrastructural localization of the prostasome-an organelle in human seminal plasma. *Upsala Journal of Medical Sciences*, 88(2): 63-80.

BRUN, J.M.; THEAU-CLÉMENT, M.; BOLET, G. (2002).The relationship between rabbit semen characteristics and reproductive performance after artificial insemination. *Animal Reproduction Science,* 70: 139-149.

BURDEN, H. P.; HOLMES, C. H.; PERSAD, R.; WHITTINGTON, K. (2006). Prostasomes-their effects on human male reproduction and fertility. *Human Reproduction Update*, 12(3): 283-292.

CAMPANILE, G.; VECCHIO, D.; DI PALO, R.; NEGLIA, G.; GASPARRINI, B.; PRANDI, A.; ZICARELLI, L.; D'OCCHIO, M.J. (2008). Delayed treatment with GnRH agonist, hCG and progesterone and reduced embryonic mortality in buffaloes. *Theriogenology*, 70:1544-1549.

CASTELLINI, C. (1996). Recent advances in rabbit artificial insemination. *In Proceedings of the 6th World Rabbit Congress, Toulouse, France,* 2: 13-26.

CASTELLINI, C.; LATTATIOLI, P. (1999). Effect of number of motile sperms inseminated on reproductive performance of rabbit does. *Animal Reproduction Science*, 57: 111-120.

CASTELLINI, C.; MOURVAKI, E.; CARDINALI, R.; DAL BOSCO, A.; DEL VECCHIO, M.T. (2008). Rabbit seminal granules: secretion site and main role, in: *Sperm Morphology and Pathology*. Ed. Research Signpost. Kerala, 45-59.

COLLODEL, G.; CARDINALI, R.; MORETTI, S.; MATTIOLI, S.; RUGGERI, S.; CASTELLINI, C. (2012). Role of rabbit prostate granules on sperm viability and acrosome reaction evaluated with different methods. *Theriogenology*, 77(5): 1021-1026.

ESTANY, J.; BASELGA, M.; BLASCO, A.; CAMACHO, J. (1989). Mixed model methodology for the estimation of genetic response to selection in litter size of rabbits. *Livestock Production Science,* 21(1), 67-75.

FABIANI, R.; JOHANSSON, J.; LUNDKVIST, Ö.; RONQUIST, G. (1995) Prolongation and improvement of prostasome promotive effect on sperm forward motility. *European Journal of Obstetrics Gynecology and Reproductive Biology*, 58: 191–198.

FARRELL, P.B.; FOOTE, R.H.; SIMKIN, M.E.; CLEGG, E.D.; WALL, R.J. (1993). Relationship of semen quality, number of sperm inseminated, and fertility in rabbits. *Journal of Andrology*, 14: 464-471.

FERNÁNDEZ, D.; VALDIVIA, A.; IRAZUSTA, J.; OCHOA, C.; CASIS, L. (2002). Peptidase activities in human semen. *Peptides,* 23(3): 461-468.

FOOTE, R.H.; SIMKIN, E.M. (1993). Use of gonadotropic releasing hormone for ovulating the rabbit model. *Laboratory Animal Science,* 43(4): 383-385.

GREENBERG, L. J. (1962). Fluorometric measurement of alkaline phosphatase and aminopeptidase activities in the order of 10< sup>− 14</sup> mole. *Biochemical and biophysical research communications*, 9(5): 430-435.

HUANG, K.; TAKAHARA, S.; KINOUCHI, T.; TAKAYAMA, M.; ISHIDA, T.; UEYAMA, H.; NISHI, K.; OHKUBO, I. (1997) Alanyl aminopeptidase from human seminal plasma: purification, characterization and immunohistochemical localization in the male genital tract. *Journal of Biochemistry*, 122: 779-787.

IRAZUSTA, J.; VALDIVIA, A.; FERNÁNDEZ, D.; AGIRREGOIIA, E.; OCHOA, C.; CASIS, L. (2004). Enkephalin-degrading enzymes in normal and subfertile human semen. *Journal of Andrology*, 25 (5): 733-739.

KAIM, M.; BLOCH, A.; WOLFENSON, D.; BRAW-TAL, R.; ROSENBERG, M.; VOET, H.; FOLMAN, Y. (2003). Effects of GnRH administered to cows at the onset of estrous on timing of ovulation, endocrine responses and conception. *Journal Diary Science*, 86: 2012-2021.

KELLY, R.W.; HOLLAND, P.; SKIBINSKI, G.; HARRISON, C.; MCMILLAN, L.; HARGREAVE, T.; JAMES, K. (1991). Extracellular organelles (prostasomes) are immunosuppressive components of human semen. *Clinical and Experimental Immunology*, 86: 550-556.

LAVARA, R.; MOCÉ, E.; LAVARA, F.; VIUDES-DE-CASTRO, M.P.; VICENTE, J.S. (2005). Do parameters of seminal quality correlate with the results of on-farm inseminations in rabbits?. *Theriogenology*, 64: 1130-1141.

LAVARA, R.; GARCÍA, M.L.; TORRES, C.; VICENTE, J.S.; BASELGA, M. (2008). Genetic parameters for semen traits of rabbit males: I. Production, morphology, and sperm head morphometry. *In Proceedings of the 9th WRSA Congress*, 153-158.

LILJA, H.; LAUREL, C.B. (1984). Liquefaction of coagulated human semen. *Scandinavian Journal of Clinical and Laboratory Investigation*, 44: 447-452.

MAWHINNEY, I.; BIGGADYKE, H.; DREW, B. (1999). Field trial of a planned breeding regiment for diary cows, using gonadotrophin-releasing hormone and prostaglandin F2. *Veterinary Record*, 145: 551-554.

MAXWELL, W.M.C.; JOHNSON, I.A. (1999). Physiology of spermatozoa at high dilution rates: the influence of seminal plasma. *Theriogenology*, 52: 1353-1362.

MINISTERIO DE AGRICULTURA, ALIMENTACIÓN Y MEDIO AMBIENTE, (2008). *Estadísticas agrarias.*
http://www.magrama.gob.es/es/estadistica/temas/estadisticas-agrarias/2008_Cunicultura_Memoria_tcm7-14332.pdf

OKADA, H.; YAMAZAKI, I.; OGAWA, Y.; HIRAI, S.; YASHIKI, M.T.; MIMA, H. (1982). Vaginal absorption of a potent luteinizing hormone releasing hormone analogue (leuprolide) in rats. I. Absorption by various routes and absorption enhancement. *Journal of Pharmaceutical Sciences,* 71: 1367-1371.

OKADA, H.; YASHIKI, M.T.; MIMA, H. (1984). Vaginal absorption of a potent luteinizing hormone-releasing hormone analogue (leuprolide) in rats III: Effect of strous cycle on vaginal absorption of hydrophilic model compounds. *Journal of Pharmaceutical Sciences,* 73: 298-302.

ONDRUŠKA, L.; PARKÁNYI, V.; RAFAY, J.; CHLEBEC, I.; XICATO, G.; TROCINO, A.; LUKEFAHR, S.D. (2008). Effect of LHRH analogue included in seminal dose on kindling rate and prolificacy of rabbits artificially inseminated. *In Proceedings of the 9th World Rabbit Congress, Verona, Italy,* 423-426.

OSADA, T.; WATANABE, G.; KONDO, S.; TOYODA, M.; SAKAKI, Y.; TAKEUCHI, T. (2001). Male reproductive defects caused by puromycin-sensitive aminopeptidase deficiency in mice. *Molecular Endocrinology*, 15 (6): 960-971.

PADULA, A.M. (2005). GnRH analogues-agonists and antagonists. *Animal Reproduction Science*, 88: 115-126.

PÉREZ-PE, R. ; BARRIOS, B. ; MUIÑO-BLANCO, T. ; CEBRIÁ, J.A. (2001). Troedsson et al, 2005Seasonal differences in ram seminal plasma revealed by partition in an aqueous two-phase system. *Journal of Chromatography B: Biomedical Science and Applications,* 760: 113-121.

PERRIER, G.; THEAU-CLÉMENT, M.; JOUANNO, M. ; DROUET, J.P. (2000). Reduction of the GnRH dose and inseminated rabbit doe reproductive performance. *World Rabbit Science*, 8: 225-230.

PETERS, A.R.; DWYER, L.; CANHAM, P.A.; MACKINNON, J.D. (2000). Effect of gonadotrophin-releasing hormone on the fertility of sows kept outdoors. *Veterinary Record*, 147: 649-652.

POIANI, A. (2006). Complexity of seminal fluid: a review. *Behavioral Ecology and Sociobiology*, 60: 289-310.

QUINTELA, L.; PEÑA, A.; VEGA, M.; GULLÓN, J.; PRIETO, M.; BARRIO, M.; BECERRA, J.J.; MASEDA, F.; HERRADÓN, P. (2004). Ovulation induction in rabbit does submitted to artificial insemination by adding buserelin to the seminal dose. *Reproduction Nutrition Development*, 44(1): 79-88.

RODRÍGUEZ, J.M.; UBILLA, E. (1988). Effect of sexual receptivity on ovulation response in rabbit does induced with GnRH. *In Proceedings of the 4th World Rabbit Congress*, 504-509.

SAEZ, F.; FRENETTE, G.; SULLIVAN, R. (2003). Epididymosomes and prostasomes: their roles in posttesticular maturation of the sperm cells. *Journal of Andrology*, 24(2): 149–154.

SMITH, P.K.; KROHN, R.I.; HERMANSON, G.T.; MALLIA, A.K.; GARTNER, F.H.; PROVENZANO, M.; FUJIMOTO, E.K.; GOEKE, N.M.; OLSON, B.J.; KLENK, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical biochemistry*, 150(1): 76-85.

STEGMAYR, B.; RONQUIST, G. (1982a) Promotive Effect on Human Sperm Progressive Motility by Prostasomes. *Urological Research*, 10: 253-257.

STEGMAYR, B.; RONQUIST, G. (1982b) Stimulation of sperm progressive motility by organelles in human seminal plasma. *Scandinavian Journal of Urology and Nephrology*, 16: 85-90

SULLIVAN, R.; SAEZ, F. (2013). Epididymosomes, prostasomes, and liposomes: their roles in mammalian male reproductive physiology. *Reproduction*, 146(1) : 21-35.

THEAU-CLÉMENT, M.; BRUN, J.M.; SABBIONI, E.; CASTELLINI, C.; RENIERI, T.; BESENFELDER, U.; FALIERES, J.; ESPARBIE, J.; SALEIS, G. (2003). Comparaison de la production spermatique de trois souches de lapins : moyennes et variabilités. *In Proceedings of the 10èmes Journées de la Recherche Cunicole, Paris*, 81-84.

THEAU-CLÉMENT, M.; BOLET, G.; ROUSTAN, A.; MERCIER, P. (1990). Comparaison de différents modes d'induction de l'ovulation chez les lapines multipares en relation avec leur stade physiologique et la réceptivité au moment de la mise à la

reproduction. *In Proceedings of the 5es Journées de la Recherche Cunicole, Paris, France,* Tome I, Communication 6.

TROEDSSON, M.H.T.; DESVOUSGES, A.; ALGHAMDI, A.S.; DAHMS, B.; DOW, D.A.; HAYNA, J.; VALESCO, R.; COLLAHAN, P.T.; MACPHERSON, M.L. POZOR, M.; BUHI, W.C. (2005). Components in seminal plasma regulating sperm transport and elimination. *Animal Reproduction Science,* 89(1): 171-186.

UTLEG, A.G.; YI, E.C.; XIE, T.;SHANNON, P.; WHITE, J.T.; GOODLETT, D.R.; HOOD, L.; LIN, B. (2003). Proteomic Analysis of Human Prostasomes. *Prostate,* 56: 150-161.

VICENTE, J.S.; LAVARA, R.; MARCO-JIMÉNEZ, F.; VIUDES-DE-CASTRO, M.P. (2011). Detrimental effect on availability of buserelin acetate administered in seminal doses in rabbits. *Theriogenology*, 76(6): 1120-1125.

VIUDES-DE-CASTRO, M.P.; VICENTE, J.S. (1997). Effect of sperm count on the fertility and prolificity rates of meat rabbits. *Animal Reproduction Science,* 46: 313-319.

VIUDES-DE-CASTRO, M.P.; VICENTE, J.S.; LAVARA, R. (1999). Effet du nombre de spermatozoïdes sur la fertilité de la semence conserve 24 heures chez le lapin. *Annales de Zootechnie,* 48: 407-412.

VIUDES-DE-CASTRO, M.P.; MARCO-JIMÉNEZ, F.; VICENTE, J.S.; NAVARRO, E.; LAVARA, R.; MOCÉ, E. (2004). Sperm kinetic parameters and differences in seminal plasma composition among two rabbit lines. *Reproduction in Domestical Animals,* poster 276.

VIUDES-DE-CASTRO, M.P.; LAVARA, R.; MARCO-JIMÉNEZ, F.; CORTELL, C.; VICENTE, J.S. (2007). Ovulation induced by mucosa vaginal absorption of buserelin and triptorelin in rabbit. *Theriogenology,* 68(7): 1031-1036.

VIUDES-DE-CASTRO, M.P.; MOCÉ, E.; LAVARA, R.; MARCO-JIMÉNEZ, F.; VICENTE, J.S. (2014). Aminopeptidase activity in seminal plasma and effect of dilution rate on rabbit reproductive performance after insemination with an extender supplemented with buserelin acetate. *Theriogenology*, 81: 1223-1228.

WILLARD, S.; GANDY, S.; BOWERS, S.; GRAVES, K.; ELIAS, A.; WHISNANT, C. (2003). The effects of GnRH administration postinsemination on serum concentrations of progesterone and pregnancy rates in dairy cattle exposed to mild summer heat stress. *Theriogenology*, 59:1799-1810.

XU, X.; POMMIER, S.; ARBOR, T.; HUTCHINGS, B.; SOTTO, W.; FOXCROFT, G.R. (1998). In vitro maturation and fertilization techniques for assessment of semen quality and boar fertility. *Journal of Animal Science*, 76: 3079-3089.

ZAPLETAL, D.; PAVLIK, A. (2008). The effect of lecirelin (GnRH) dosage on the reproductive performance of nulliparous and lactating rabbit does. *Animal Reproduction Science*, 104: 306-315.

ZHU, J.; ZU, X.; COSGROVE, J.R.; FOXCROFT, G.R. (2000). Effects of semen plasma from different fractions of individual ejaculates on IVF in pigs. *Theriogenology*, 54: 1443-1445.

8. ANNEXES

8.1 Annex I: Aminopeptidase activity evolution regarding age and male.

Only activity evolution of males with more than 10 samples available is represented to ensure that trends are representative enough.

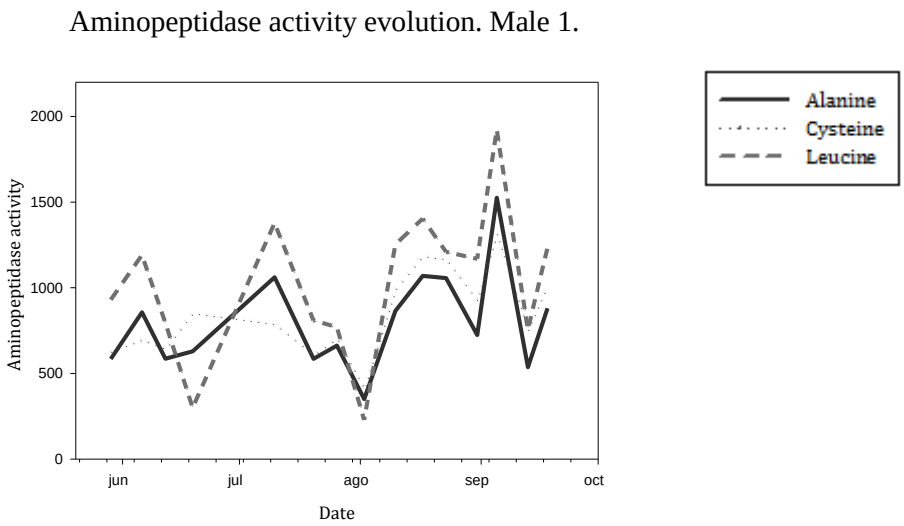

Figure 1. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 1.

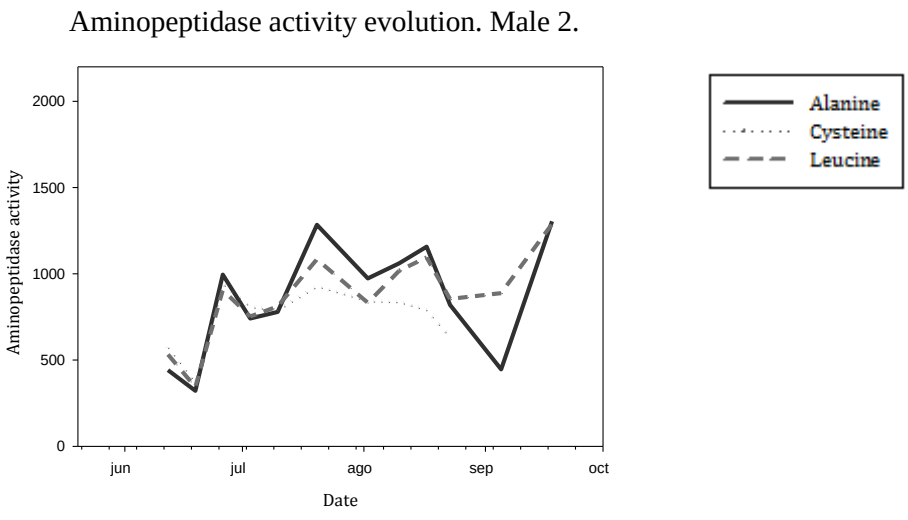

Figure 2. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 2.

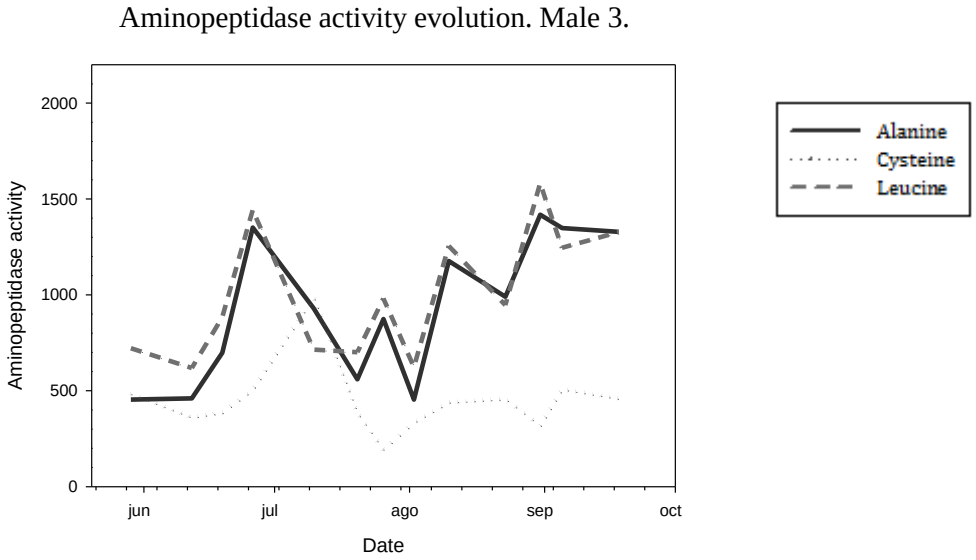

Figure 3. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 3.

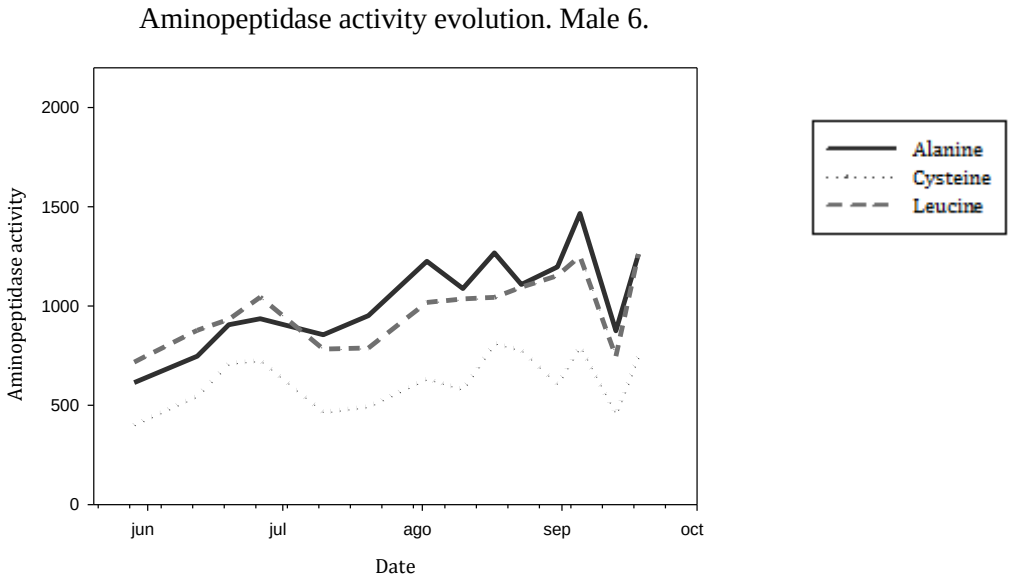

Figure 4. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 6.

Aminopeptidase activity evolution. Male 7.

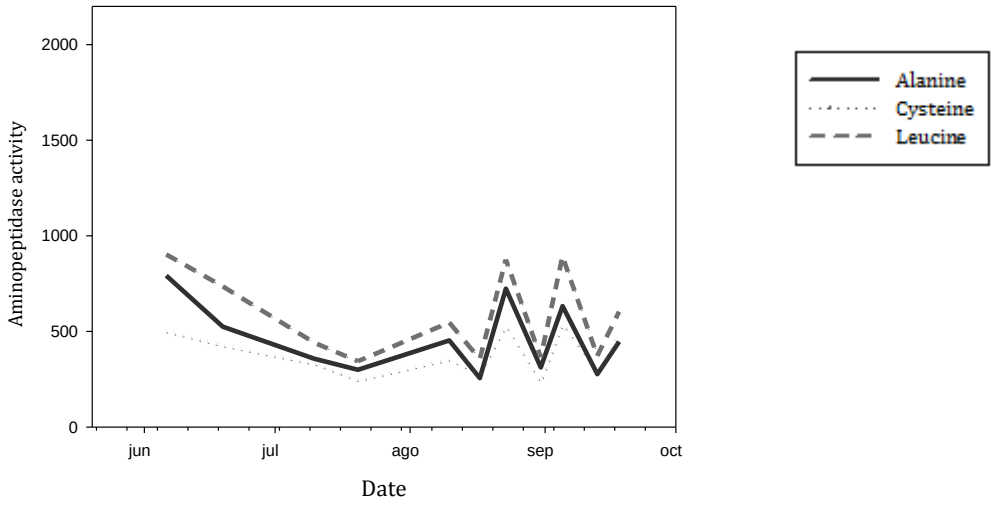

Figure 5. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 7.

Aminopeptidase activity evolution. Male 8.

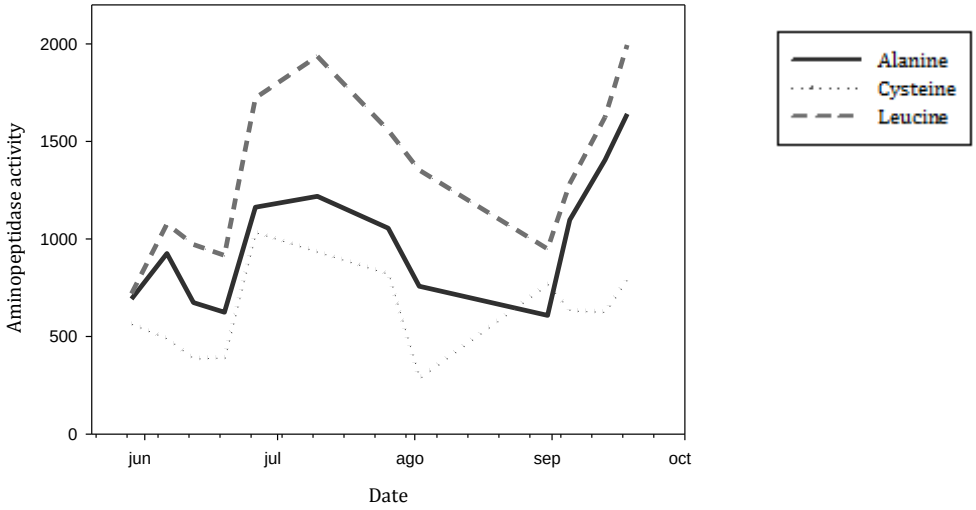

Figure 6. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 8.

Aminopeptidase activity evolution. Male 9.

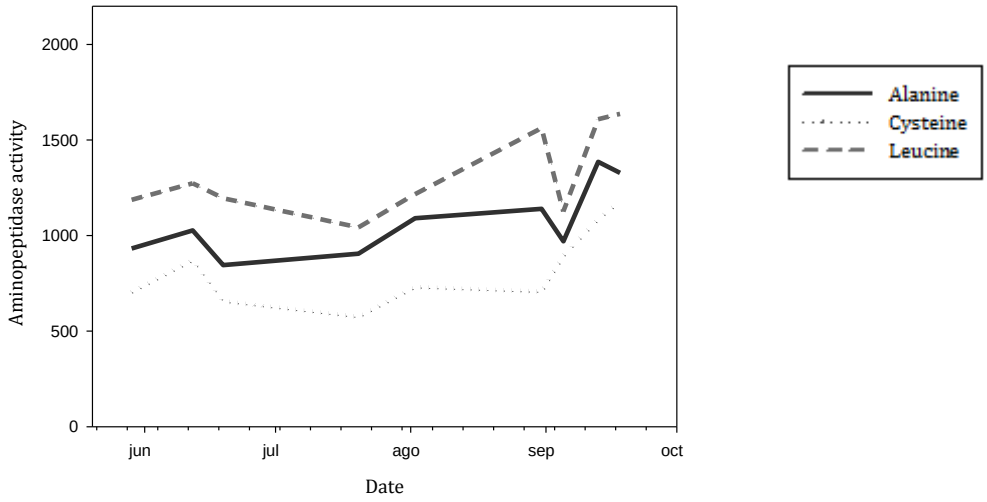

Figure 7. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 9.

Aminopeptidase activity evolution. Male 10.

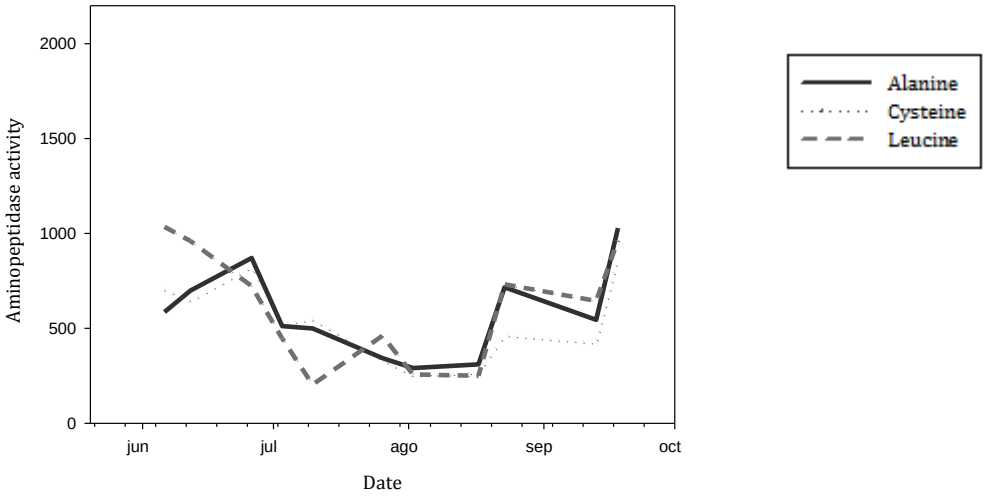

Figure 8. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 10.

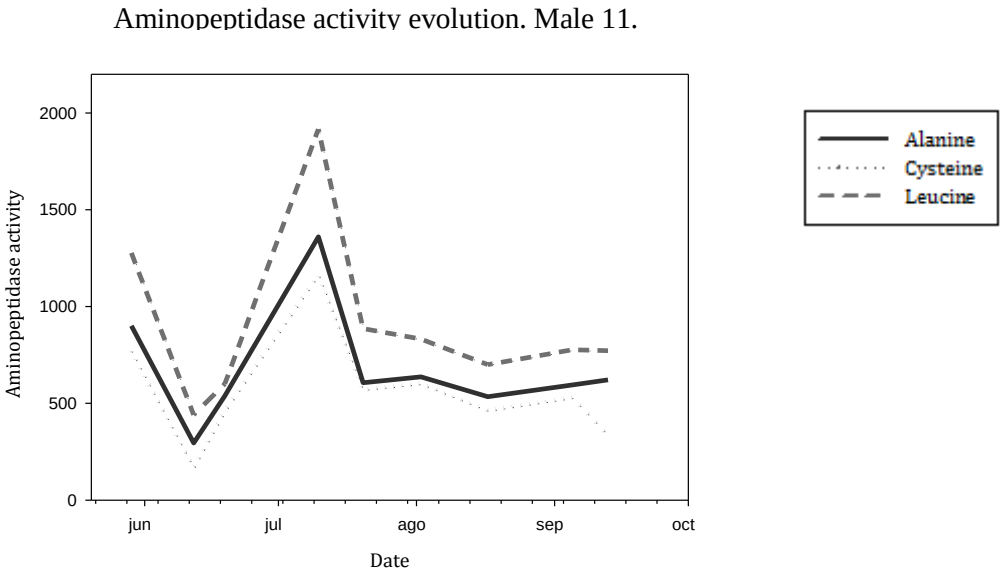

Figure 9. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 11.

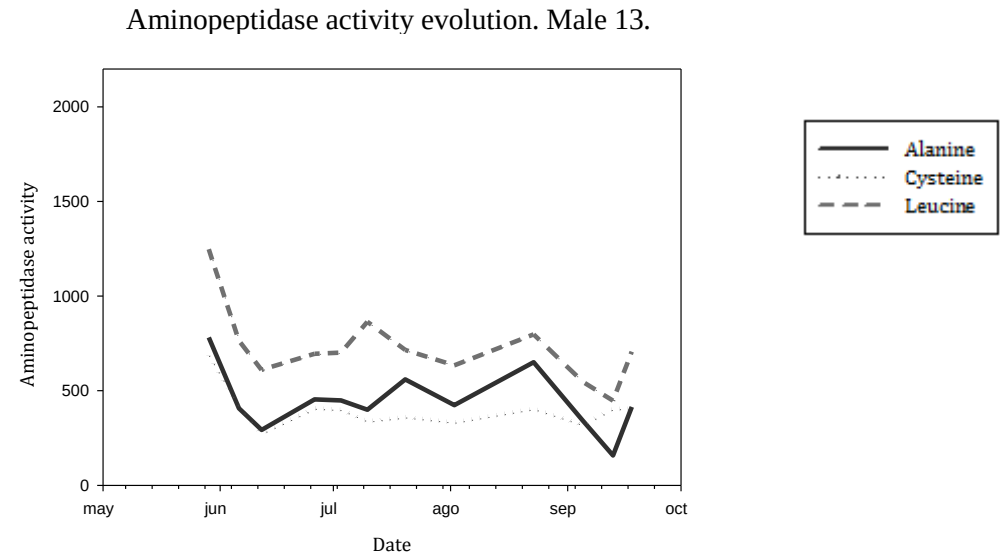

Figure 10. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 13.

Aminopeptidase activity evolution. Male 14.

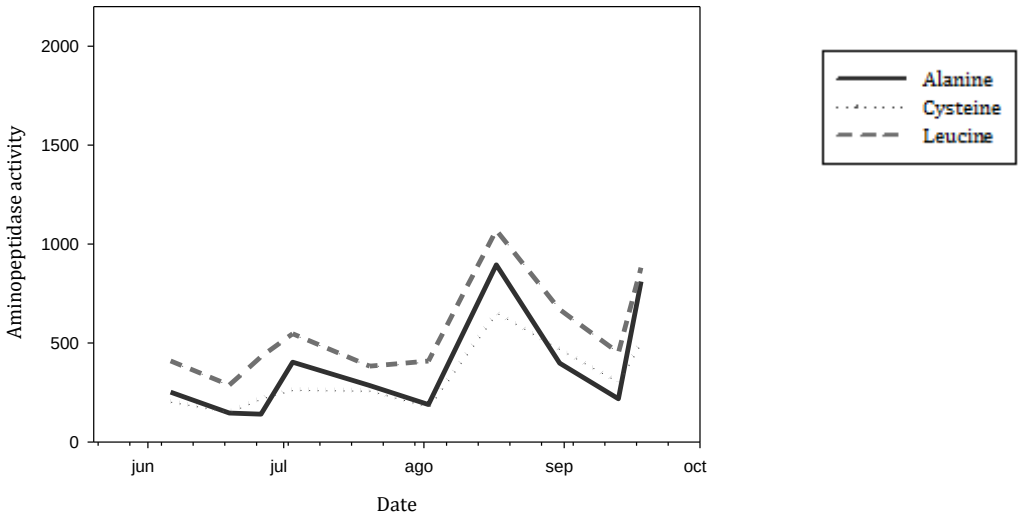

Figure 11. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 14.

Aminopeptidase activity evolution. Male 15.

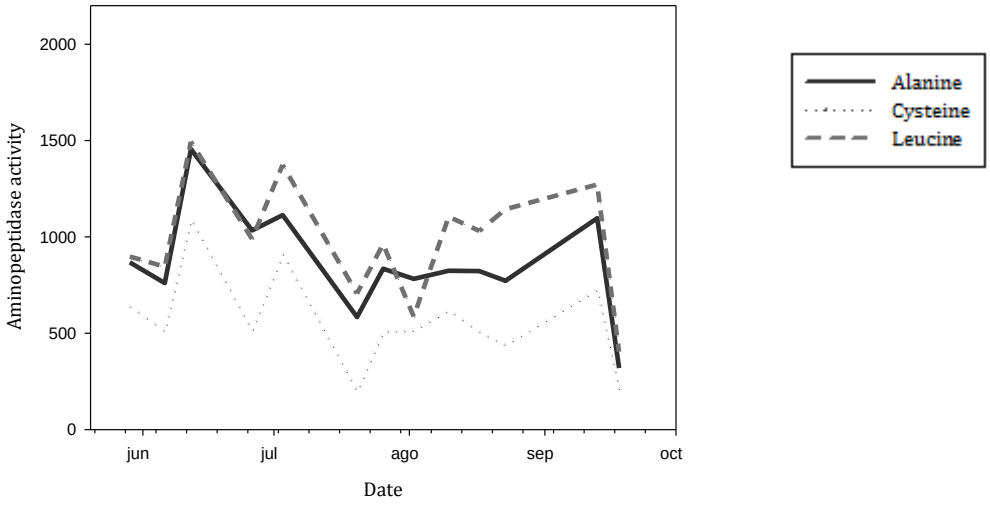

Figure 12. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 15.

Aminopeptidase activity evolution. Male 16.

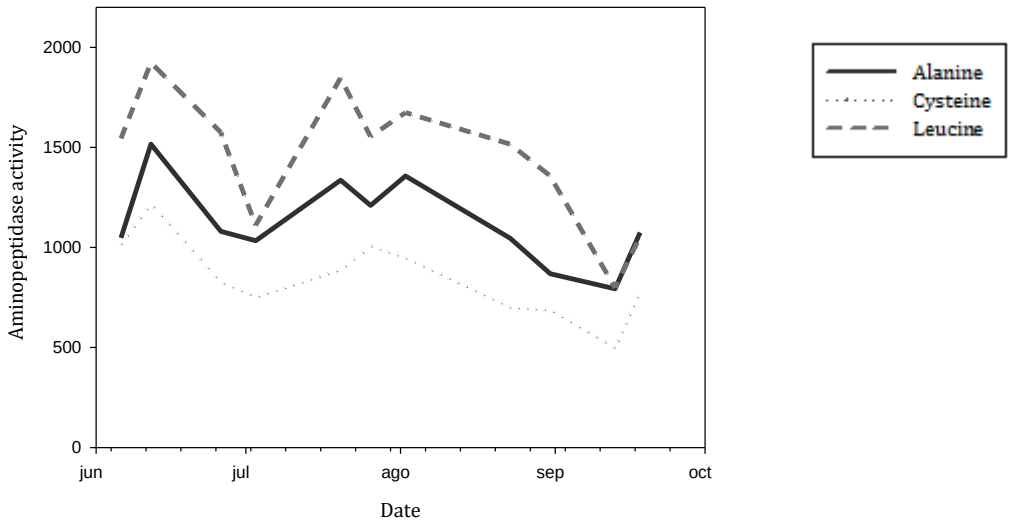

Figure 13. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 16.

Aminopeptidase activity evolution. Male 17.

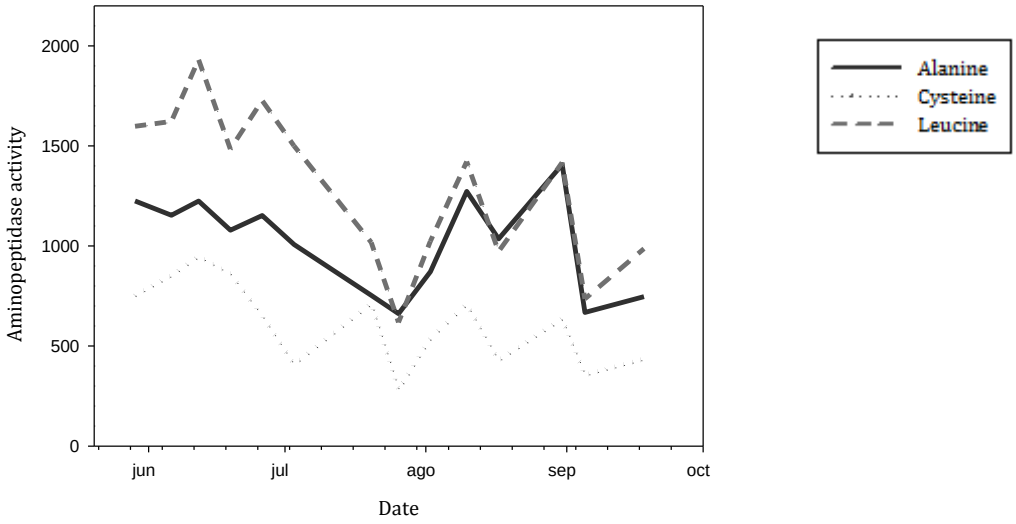

Figure 14. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 17.

Aminopeptidase activity evolution. Male 18.

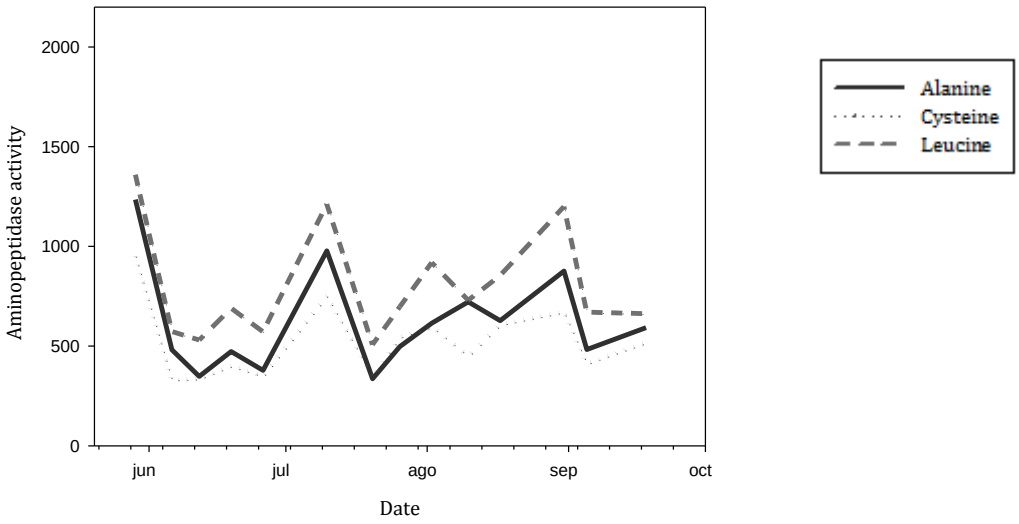

Figure 15. Aminopeptidase activity evolution (pmol β-naphthylamide/mg of protein/min). Male 18.