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Sex-based differences in the enteroendocrine system and food intake regulation due to dietary protein replacement by *Alphitobius Diaperinus* in rats

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ABSTRACT

The rising global demand for animal protein, coupled with the environmental impact of conventional livestock, has sparked interest in sustainable alternatives. Insect-derived proteins, particularly from *Alphitobius diaperinus* (lesser mealworm), offer high nutritional value, good digestibility, and a minimal ecological footprint. Enteroendocrine hormones such as ghrelin, peptide YY (PYY), cholecystokinin (CCK), and glucagon-like peptide-1 (GLP-1) are key regulators of appetite, satiety, and energy homeostasis, responding dynamically to the composition and quality of ingested nutrients. Given the increasing prevalence of protein malnutrition in aging population and the metabolic challenges posed by obesogenic diets, this study aimed to investigate whether the enteroendocrine-modulating effects previously observed with acute *A. diaperinus* administration are sustained when utilized as the sole dietary protein source. Furthermore, we evaluated sex-specific responses under both standard and high-fat diet conditions in rats.

Our findings demonstrate that *A. diaperinus*-based diets can alter caloric intake and elicit differential enteroendocrine secretion patterns depending on the sex and metabolic status of the animal, suggesting context-specific regulatory effects on energy balance and gut hormone responses.

Together, these results enhance our understanding of how insect-based proteins interact with nutrient-sensing pathways and may inform the development of targeted nutritional strategies to support healthy aging, metabolic health, and environmental sustainability.

1. Introduction

The global demand for animal protein is expected to rise significantly by 2050, driven by population growth and dietary shifts. However, conventional livestock production is increasingly unsustainable, accounting for 11–20% of global greenhouse gas emissions and uses 70% of agricultural land. Consequently, there is an urgent need for alternative protein sources (Dourmad et al., 2014). Insect-derived proteins, particularly from *Alphitobius diaperinus* (lesser mealworm), have emerged as a promising solution due to their nutritional composition, sustainability, and low environmental impact (Sousa et al., 2020). Notably, frozen, paste, dried and powder forms of *A. diaperinus* larvae have been included in the list of authorised insect-based novel foods in the European Union since 2023 (Meijer et al., 2025).

Recent studies have demonstrated that *A. diaperinus* protein hydrolysates have significant bioactive properties, including antioxidant and antihypertensive activities. This makes them suitable for integration into the food industry (Sousa et al., 2020). Additionally, the structural properties of *A. diaperinus* proteins, such as their high α-helix and β-sheet content, indicate stable conformations and high digestibility rates (up to 84.04%), comparable to conventional protein sources like casein (Ma et al., 2025).

We have previously shown that equivalent protein loads of beef, insect (*A. diaperinus)* and almond exerted distinct enterohormone secretome, leading to differences in food intake in rats (Miguéns-Gómez et al., 2020). Moreover, acute administration of *A. diaperinus* protein hydrolysates transiently increased food intake in rats during the first week of exposure, likely mediated by ghrelin modulation (Miguéns-

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Gómez et al., 2023). Ex vivo experiments revealed a 40% reduction in acute ghrelin secretion in jejunal tissues treated with digested *A. diaperinus* protein, coupled with a compensatory upregulation of ileal ghrelin receptor (GHSR1a) mRNA expression after chronic exposure (Miguéns-Gómez et al., 2023). These findings suggest that insect-derived proteins interact differently than beef or plant proteins with enteroendocrine pathways through amino acid composition-specific mechanisms.

An increase in food intake, as it has been observed after an acute load of *A. diaperinus*, is of particular interest in conditions such as aging. It is well documented that low calorie and protein intakes in older adults contribute to a cascade of adverse health outcomes that significantly diminish quality of life (Amarya et al., 2015). In the elderly, the age-related reduction in muscle protein synthesis, a phenomenon compounded by anabolic resistance, means that the conventional protein recommendations often fall short of the requirements to preserve muscle mass and functional capacity (Nowson & O'Connell, 2015). Contributing factors such as diminished taste and smell, compromised dental health, and gastrointestinal changes further impede the consumption and effective utilization of traditional protein sources, creating a vicious cycle that exacerbates malnutrition (Clegg & Williams, 2018)(Kaur et al., 2019). These challenges have prompted interest in alternative protein sources with enhanced digestibility, optimized amino acid profiles, and improved bioavailability, which could stimulate muscle protein synthesis more effectively and counteract the deleterious effects of catabolism in aging populations (Baum et al., 2016). Moreover, evidence suggests that a more evenly distributed intake of high-quality protein throughout the day can further mitigate muscle degradation and reduce the risk of frailty-associated complications (Nowson & O'Connell, 2015)(Kaur et al., 2019). It is therefore necessary to analyse whether the effects of an acute load of *A. diaperinus* would be reproduced when it is administered as protein replacement in the diet.

Conversely, effects promoting food intake would result in deleterious situations in contexts of overconsumption, as occurs with Western diets. Therefore, it is also important to analyse the effects of protein replacement by *A. diaperinus* under obesogenic conditions. A study has already been performed on male mice fed a high-fat diet. Mice under 8 weeks of age were switched to a high-fat diet in which the protein was replaced by 50% or 100% *A. diaperinus* protein, finding no effects on body weight or food intake were found (Kang et al., 2023). Given that previous results suggest that the effects of *A. diaperinus* are mediated by the modulation of the enteroendocrine system (Miguéns-Gómez et al., 2023), and considering that obesogenic diets induce alterations in the enteroendocrine system in rodents (Duca et al., 2013)(Gribble & Reimann, 2019), it is necessary to carry out a more thorough study of the effects of diets based on *A. diaperinus*.

In this study, we aimed to analyse whether the previously observed modulation of the enteroendocrine system produced by a preload administration of *A. diaperinus* was also observed when the insect was used as the only source of protein. In addition, we analysed whether the dietary replacement resulted in different effects in males and females, under both standard and obesogenic diet conditions.

2. Materials and methods

2.1. Chemicals and reagents

Lesser mealworm or buffalo (*A. diaperinus* larvae powder) was provided by Ynsect (Évry-Courcouronnes, France). Chemicals, D-glucose, D-mannitol, amino acids, aprotinin, protease inhibitor cocktail (complete™ ULTRA Tablets, Roche) and foetal bovine serum were purchased from Sigma-Aldrich (Madrid, Spain). Amastatin was from Enzo Life Sciences (Madrid, Spain), and glutamine, penicillin, streptomycin and Matrigel were from Lonza (O Porriño, Spain).

2.2. Experimental diets

The experimental diets were provided by IRTA. To ensure isoproteic conditions, all diets used a purified diet as a base (Envigo++, Barcelona, Spain) that was designed for the addition of casein (as a control protein source), freeze dried beef, or insect powder as primary protein sources, depending on the experimental group. The composition of the basal mixes was carefully designed to adjust macronutrients and micronutrients (minerals and vitamins) for a complete diet with 14% protein from the respective sources and balanced carbohydrate, fat, and fatty acid contents, to achieve nutritional equivalence among the diets (Supplementary Table 1). Cocoa butter, soybean oil, olive oil, and safflower oil were used to balance saturated, monounsaturated and polyunsaturated fatty acids among diets. The final mixes were used to produce the pellet feed for rats. Samples were elaborated using a pilot plant co-rotating and intermeshing twin-screw extruder (Evolum 25, Clextral, Firminy, France) with a 24-screw length to diameter ratio. A twin-screw volumetric feeder was used for feed dosing and a pump for the water dosing. The outlet nozzle used was 5 mm. A cutter with a Clextral EX21 four-blade cutter, with adjustable cutting speeds between 300 and 1500 rpm, was attached to the extruder. After the extrusion process, the samples were dried in an oven at 110 °C during 20–30 min till the target moisture content (<10%). Finally, the pellets were coated with sunflower oil.

The cafeteria (CAF) diet consisted of energy-dense, palatable food items—including milk with added sugar, sausages, carrots, bacon, biscuits with pâté, and muffins—which were provided ad libitum to promote hyperphagic behaviour.

2.3. Animals

We used 46 female Wistar rats (10 weeks old, 201–246 g) and 50 male Wistar rats (10 weeks old, 400-500 g) purchased from Janvier (Castellar del Vallés, Spain). These animals were housed at the animal housing facility of the University Rovira i Virgili under standard conditions (22 °C and 55% humidity). Upon arrival, the animals were housed in pairs for a week and then individually in animal quarters for another week to get them used to the voluntary oral administration. The rats had free access to food: a standard Teklad diet (Cat No: Teklad 2014, Envigo++, Barcelona, Spain) containing (by energy) 14.3% protein, 4% fat, 48% carbohydrates 22% fibre and tap water. The room temperature was kept at 22 °C with a 12 h light/12 h dark cycle (lights from 6:00 a.m. to 6:00 p.m.). After the acclimatization period, animals were randomly assigned to two main experimental groups: a standard diet group and an obesogenic group ($n = 24$, $n = 18$ respectively). During the first eight weeks, all animals were fed a standard chow diet ad libitum until the specific diets started. The obesogenic group also received a cafeteria diet to induce hyperphagia and promote the development of an obesity-prone metabolic phenotype. After these eight weeks, animals were redistributed into specific dietary intervention groups (Fig. 1). The standard diet group was divided int o three subgroups ($n = 8$ per group), according to the primary protein source in their diet:

- **Control**: animals received a custom-formulated diet, casein-based.
- **Beef**: animals were fed a diet formulated with beef as protein source.
- **Insect**: animals received a diet in which *A. diaperinus* (insect protein) was the protein source.

Concurrently, animals in the obesogenic group were divided into two subgroups ($n = 9$):

- **CAF**: animals continued the cafeteria diet in combination with the control diet containing casein as the protein source.
- **CAF—I**: animals received the cafeteria diet along with the insect diet in which *A. diaperinus* was the protein source.

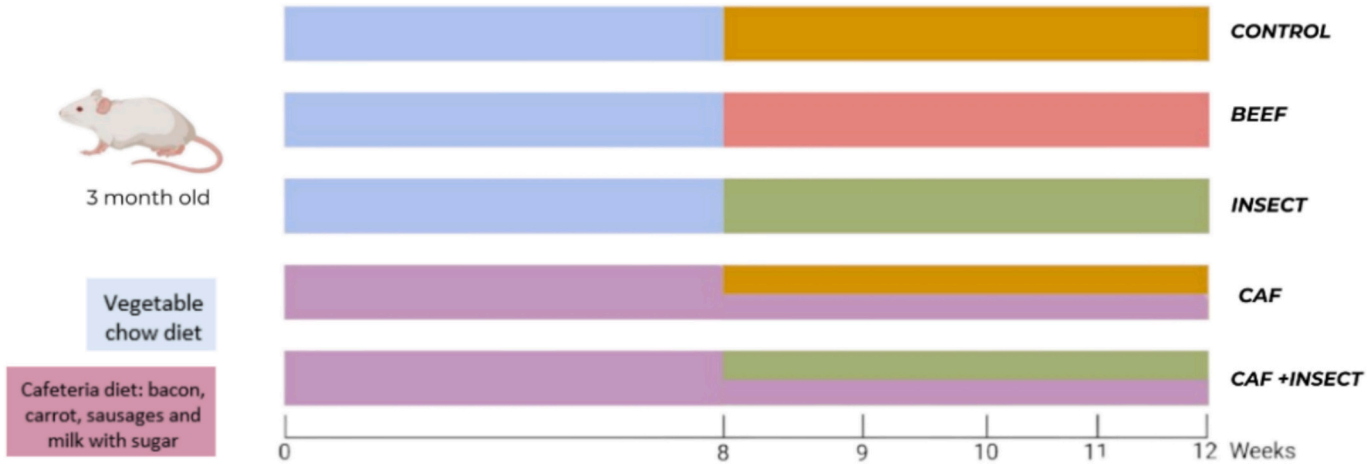

Fig. 1. Experimental design. Dietary intervention groups were divided at the end of week 8 according to the primary protein source (casein, beef or insect respectively). For obesogenic induced metabolism (Cafeteria diets) specific diet were divided into two group: casein and insect group respectively.

The animals had free access to food during the entire experiment. Body weight was measured weekly throughout the experiment. Food intake was measured for 20 h daily in the first week, then, after that, two days at the middle of the experiment and before sacrifice (week 4 from when the specific diets started). At day 23 from specific diets start, food was removed at 7 a.m. and at 15 p.m. plasma was collected from the saphenous vein and immediately frozen for further analysis of the biochemical parameters under fasting conditions (8 h of fasting). After the animals were sacrificed by beheading (between 9 and 12 a.m.), without previous fasting, the blood was collected, from the cervical vessels, using EDTA covered tubes (Deltalab, Barcelona, Spain) as anticoagulant.

Plasma was obtained by centrifugation at 4500 ×*g* for 15 min at 4 °C and stored at −80 °C until further analysis. Three types of aliquots were prepared: one aliquot (1000 μL) was supplemented with protease inhibitor cocktail to enable the analysis of circulating anorexigenic hormones (GLP1, PYY, CCK); a second aliquot (500 μL) was collected in tubes containing hydrochloric acid (final concentration: 0.1 M) together with a protease inhibitor cocktail, specifically for the quantification of circulating ghrelin; and a third aliquot containing the rest of the sample was reserved for biochemical studies.

The intestine was excised, rinsed with ice-cold phosphate-buffered saline (PBS), and segmented anatomically. Specific segments of the duodenum and jejunum were dissected and used immediately for ex vivo functional experiments. The remaining intestinal tissue was divided into sections, rapidly snap-frozen in liquid nitrogen, and stored at −80 °C for subsequent analyses. Additionally, distinct white adipose tissue depots were dissected and weighed for comparative analyses. All procedures were approved by The Animal Ethics Committee of University Rovira i Virgili (Tarragona, Spain) (project no. 11817, Generalitat de Catalunya, Department of Climate Action, Food and Rural Agenda).

2.4. Ex vivo experiments with intestinal segments

Two ex vivo experiments were conducted using the intestinal segments from the animals that had been fed the diets with the different protein sources (*A. diaperinus*, beef and casein as control): i) an experiment using duodenum segments to analyse the secretory response of CCK and ghrelin; and ii) an analysis of the jejunum secretory response to study GLP-1 and PYY secretion.

After washing the intestinal tube, the outer muscular layer of the corresponding intestinal segments was removed from the serosa layer with a scalpel. The tube was then cut longitudinally and tissue circles, with a diameter of 5 mm, were obtained using a biopsy punch. The sample was kept at a low temperature with cold PBS buffer and an ice bath throughout the procedure. We then began the secretion study. Each circular segment of distal jejunum was placed in a well (48-well plate) containing 0.4 mL of Krebs–Ringer bicarbonate (KRB) buffer with 10 mM D-mannitol, pre-warmed to 37 °C for 15 min. After this pre-incubation period, the buffer was replaced with KRB buffer containing 10 mM glucose to study baseline secretion, or by pre-warmed Peptone solution dissolved in KRB buffer containing 10 mM glucose to analyse the secretory response of this segment to acute hormone stimulation. The incubation period was 30 min or 60 min (depending on the type of hormone under study). All the buffers used to incubate the tissue were previously oxygenated (95% O2, 5% CO2) for at least 15 min. Three replicates of each treatment were performed for each rat. Two of them were used to study basal enterohormone secretion and one was used to study the stimulated secretory response using Peptone. All the treatments contained a cocktail of protease inhibitors, 100 KIU aprotinin, 10 μM Amastatin and 0.1% fatty acid free BSA. After the incubation period, the medium was collected in different aliquots and stored at −80 °C for enterohormone quantification.

2.5. Biochemical parameters and enterohormones quantification

Plasmatic parameters were measured by colorimetric methods using commercial kits according to the manufacturer's instructions. For insulin, an ELISA kit (catalogue no. EZRMI-13 K) from Millipore (Madrid, Spain) was used. For measured glucose, triglycerides and cholesterol we used a kit from QCA (Amposta, Spain). Non-esterified fatty acid (NEFA) levels were quantified using the NEFA Standard Kit (Ref. 270–77,000; FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany). ELISA kits for measuring total ghrelin (catalogue no. EZGRT-91 K) and total GLP-1 (catalogue no. EZGLPT1-36 K) were acquired from Millipore (Billerica, MA, USA). For PYY and CCK, ELISA kits (catalogue no. FEK-059-03 and catalogue no. EKE-069-04 respectively) were purchased from Phoenix Pharmaceuticals, INC.

Absorbance measurements were performed using an Eon Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA). For PYY quantification were carried out using a Varioskan™ LUX Multimode Microplate Reader (Thermo Scientific, Waltham, MA, USA).

2.6. Quantitative real-time RT-PCR analysis

Total RNA and cDNA were obtained by TRIzol™-chloroform method (Fisher Scientific) from ileum and duodenum samples (approximately 60 mg of tissue) (Ginés et al., 2019). Quantitative PCR amplification was performed using specific TaqMan probes from Applied Biosystems (Waltham, USA): Rn00562293_m1 for *Gcg* to determine GLP-1, and

Rn00821417_m1 for the ghrelin receptor gene, also known as Growth Hormone Secretagogue Receptor (*Ghsr*). The relative expression of each gene was compared with the control group using the 2-ΔΔCt method, with PPIA gene expression (Rn00690933_m1) as a reference.

2.7. Statistical analysis

The results are expressed as the mean ± the standard error of the mean (SEM). The sample size (n) for each variable is indicated in the corresponding figure description. The ROUT method was used to identify outliers (Q = 1), and a Shapiro Wilk test was performed to study sample normality. Depending on the normality of the samples, One-way ANOVA or the Kruskal–Wallis test was used to compare the treatments under standard conditions. For the obese group, *t*-tests were used to compare the two groups to determine whether insect supplementation influences the metabolism induced by obesity. *p* values < 0.05 were considered statistically significant. Additionally, a multivariate statistical analysis and a two-sided t-test for Pearson's correlation were also perform. Calculations were performed using IBM SPSS statistics v29 (IBM Corp. IBM SPSS Statistics for Windows, Version 29.0. Armonk, NY: IBM Corp;, 2022., n.d.) and R studio 4.1.2 (*R Core Team, Foundation for Statistical Computing*, 2023).

3. Results

3.1. Dietary protein replacement by A. diaperinus does not affect body weight gain, although it has slightly different effects on food intake according to the animal's metabolic phenotype and sex

Body weight was evaluated weekly throughout the experiment. The body weight was higher in males than in females from the first week of treatment onwards, as expected. No significant differences in body weight were observed among the groups administered diets with different specific protein sources (Fig. 2). Cafeteria diet-fed animals showed an increased body weight compared to non-cafeteria-fed animals, and this was not modified when cafeteria is supplemented with the insect-based diet. In agreement, adiposity did not differ among the different experimental groups. The visceral adiposity was measured in Table 1. Global health status was assessed through the analysis of biochemical plasmatic parameters. The levels of these parameters at

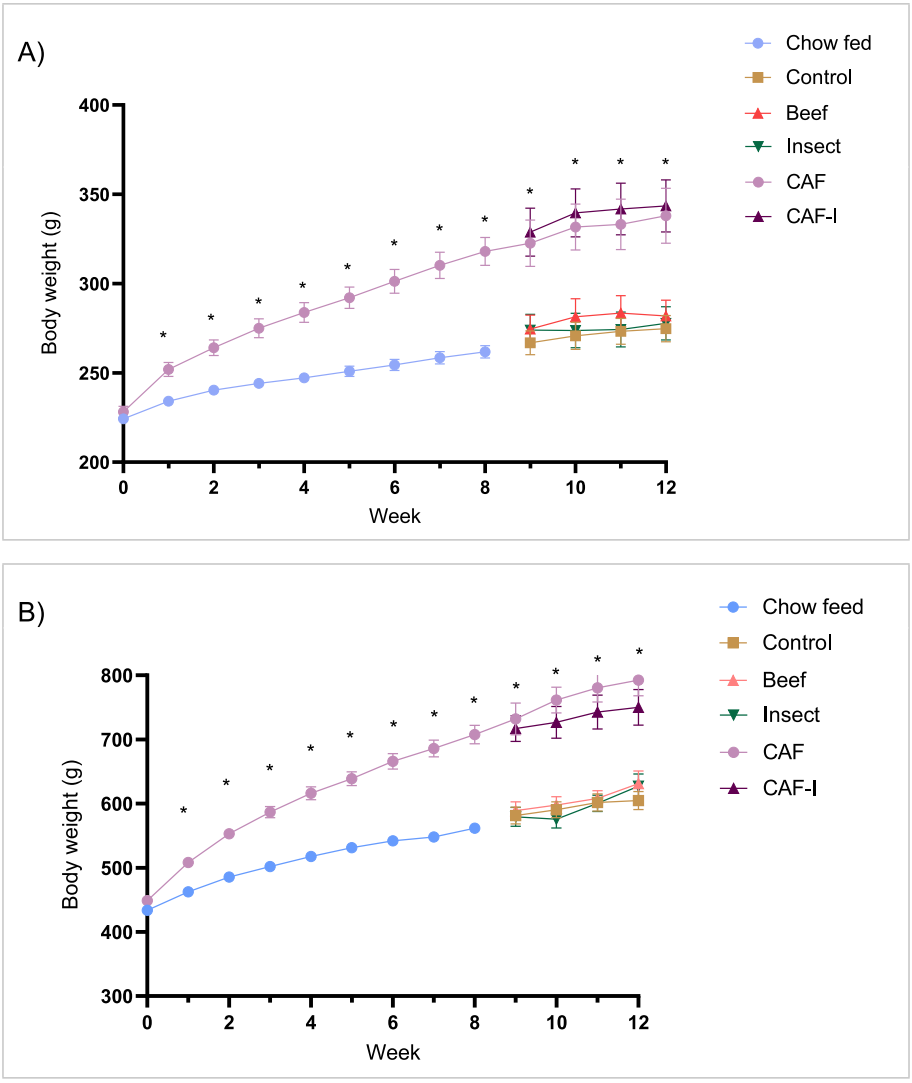

Fig. 2. Females (A) and males (B) body weight during the experiment. Results are presented as the mean ± s.e.m. (n = 6–8). * Shows differences between standard and obesogenic groups using one-way ANOVA, Tukey's multiple comparison test from week 9 to 12 (p value <0.05) and two-way ANOVA (factors: week and diet), Bonferroni multiple comparison test from week 0 to 8 (p value <0.05). No differences were found between Control, Beef and Insect groups neither between CAF and CAF-I using one-way ANOVA and Student t-test (*p* value <0.05) respectively. The ROUT and Shapiro Wilk methods was used to identify outliers (Q = 1) and study the normality respectively.

Table 1
Animals' visceral adiposity measured at the end of the experiment. Results are presented as the mean ± s.e.m. (n = 6–8).

	Control	Beef	Insect	CAF	CAF- I
Females	16.28 ± 1.45	17.09 ± 1.71	16.66 ± 0.92	31.24 ± 3.11	30.72 ± 3.40
Males	34.29 ± 3.11	40.69 ± 2.23	37.89 ± 1.91	73.82 ± 5.82	66.91 ± 5.39

sacrifice were mostly not statistically different in relation to diet sources. Only triglycerides showed significantly higher levels in the beef group of females and lower levels in the cafeteria animals under insect diet. And insect diet, under cafeteria situation produced lower cholesterolemia in males. In a fasting situation, glucose and insulin levels remained unchanged. Moreover, the HOMA-IR index was also not modified (Table 2).

Food intake was measured at different time points during the experiment. We found differences in the evolution of energy intake, with the obesogenic groups undergoing an increase in total energy intake compared with the control group, as expected. Furthermore, baseline sex-related differences in total caloric intake were observed. When analysing treatment effect, females fed an insect-based diet had a decrease (p value = 0.04) in total energy consumption at the end (day 28) of the experiment compared to the control group in a healthy metabolic rat model. Interestingly, these effects disappeared in an obesogenic induced condition and were also not found in male rats (Fig. 3).

Feed efficiency (FE) was also measured as it provides valuable insight into the animals' metabolic efficiency of using energy under different dietary interventions. Females showed a slight increase in FE in the insect group, although there were no statistical differences between groups in standard or obesogenic conditions. Males showed a different profile, although again no statistically significant differences were found. (Fig. 4).

Due to sex-specific responses to diet, an unsupervised multivariate statistical analysis was conducted to examine the simultaneous variation of physiological peripheral parameters: Body weight increase, feed efficiency, food intake, Kcal intake, protein intake, adipose tissue, biochemical release (Cholesterol, Triglycerides, Insulin, Glucose, HOMA-IR, NEFAs) and peripheral circulating hormones (Glucagon, CKK, PYY, Total GLP-1), and how sample variability could be strongly influenced by sex. Fig. 5 reveals, in a Principal component analysis (PCA), clear sex-related differences, which were strongly influenced by circulating hormones and biochemical markers. PCA disaggregated per diets also show this sex distinction (Fig. 1 Supplementary materials).

To further investigate the impact of diet on the enteroendocrine system and its relationship with physiological status, plasma concentrations of enterohormones were measured at sacrifice (Table 3). Under standard dietary conditions, insect-based protein did not induce detectable alterations in enterohormone levels. In contrast, the beef-based diet tended to reduce plasma ghrelin concentrations relative to the insect-based diet. In males, the cafeteria diet significantly increased plasma PYY levels (t-test, $p < 0.05$ vs. control), and this effect was not altered when beef protein was replaced by insect protein.

We next assessed whether plasma enterohormone levels correlated with physiological parameters associated with diet. As shown in Fig. 6, these correlations were sex dependent. In males, plasma PYY levels were positively correlated with both body weight and adipose tissue mass, whereas no such correlations were observed in females.

3.2. Jejunal enteroendocrine secretion varies by dietary protein source and sex

As enterohormones seem to play a crucial role in modulation of physiological status, we then tested the effects on the secretion of enterohormones in the different intestinal segments, both in basal conditions and stimulated with peptone, which is a protein hydrolysate.

CCK secretion was analysed in the duodenum. Table 4 shows that there were no differences in the secretion of these enterohormones at basal conditions in the healthy or obesogenic conditions, neither in females nor in males. The orexigenic hormone ghrelin was also tested in the duodenum, where it showed no differences in basal secretion in any of the conditions (Table 4).

Next, GLP-1 was assayed in the central jejunum. We found that basal GLP-1 secretion measured in jejunum explants obtained at sacrifice was higher in females fed insect protein compared to both the control and beef groups. There were however no effects on stimulated secretion. Interestingly, in males, the opposite basal effect was found, while stimulated secretion could not be analysed. (Table 4).

PYY was also assessed in the jejunum. Table 4 shows that there were no significant differences when comparing the three protein sources (Anova test). However, insect-fed rats showed lower basal PYY than beef-fed rats (significant when applying a t-test, p = 0.019), in agreement with the effects of GLP-1 in males. The stimulated PYY levels did not differ among the two protein sources in males, while in females it could not be analysed. The cafeteria diet led to a reduced basal PYY secretion, which was not affected by an insect-based diet.

Table 2
Biochemical parameters of male and female rats at week 4 of the experiment (sacrifice) and after a fasting period (last week of the experiment). Results are presented as the mean ± s.e.m. (n = 6–8). One-way ANOVA or the Kruskal-Wallis test was used (depending on sample normality) to evaluate significance between standard groups ($P < 0.05$). CAF and CAF-I were analysed and compared separately using a t-test analysis. The Grubbs method was used to identify outliers (Alpha = 0.05), and the Shapiro-Wilk test was performed to analyse normality. Different letters indicate statistical differences between diets (standard groups). * Indicates statistical differences through obesogenic groups.

		Females					**Males**				
		Control	**Beef**	**Insect**	**CAF**	**CAF- I**	**Control**	**Beef**	**Insect**	**CAF**	**CAF-I**
Sacrifice (unfasted)	Glucose (mM)	7.18 ± 0.37	6.83 ± 0.19	6.96 ± 0.19	7.78 ± 0.24	7.45 ± 0.12	5.93 ± 0.25	6.08 ± 0.11	6.15 ± 0.16	7.64 ± 0.36	7.06 ± 0.15
	NEFAs (mM)	0.55 ± 0.04	0.59 ± 0.05	0.57 ± 0.05	0.67 ± 0.04	0.57 ± 0.06	0.73 ± 0.04	0.75 ± 0.05	0.78 ± 0.05	1.09 ± 0.13	1.04 ± 0.04
	Triglycerides (mM)	0.82 ± 0.11**a**	1.46 ± 0.16**b**	1.09 ± 0.1**ab**	1.86 ± 0.40	1.17 ± 0.13*	1.09 ± 0.09	1.15 ± 0.08	1.24 ± 0.11	2.33 ± 0.30	1.77 ± 0.22
	T. Cholesterol (mM)	1.99 ± 0.12	1.96 ± 0.25	2.14 ± 0.09	2.44 ± 0.30	2.65 ± 0.18	0.80 ± 0.10	0.69 ± 0.08	0.62 ± 0.06	0.91 ± 0.13	0.56 ± 0.07*
8 h Fasted	Glucose (mM)	5.59 ± 0.18	5.90 ± 0.41	5.97 ± 0.38	5.95 ± 0.20	6.33 ± 0.38	9.92 ± 1.07	8.67 ± 0.41	8.41 ± 0.40	9.24 ± 0.51	10.13 ± 0.39
	Insulin (ng/ mL)	7.47 ± 1.16	5.94 ± 0.53	4.81 ± 0.68	8.23 ± 0.60	8.10 ± 1.02	9.40 ± 1.03	9.91 ± 0.62	9.30 ± 0.93	14.76 ± 1.83	14.31 ± 1.23
	HOMA- IR	47.14 ± 8.51	49.24 ± 8.52	42.14 ± 11.37	57.26 ± 6.15	58.64 ± 9.13	39.68 ± 9.38	50.66 ± 9.91	37.59 ± 6.21	58.83 ± 9.68	53.76 ± 9.50

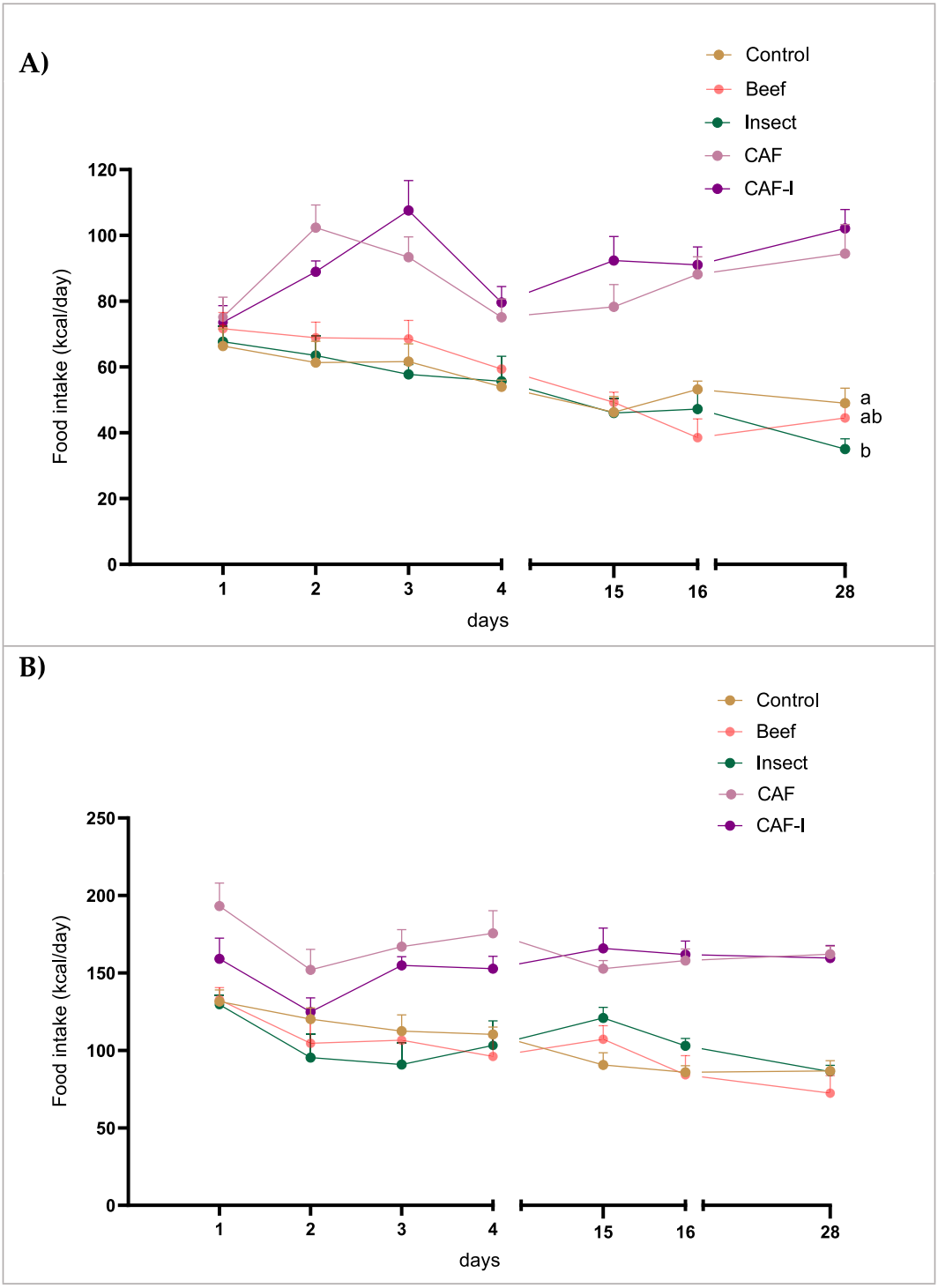

Fig. 3. Food intake (kcal/day) of 7 different days during the experiment are shown in this graph. A) Females and B) Males. Results are presented as the mean ± s.e.m. (n = 6–8). Statistical analyses were performed using Kruskal-Wallis test between standard groups (Control, Beef, Insect group). In female rats, significant differences between diets were observed and are also indicated by different letters ($P < 0.05$). CAF and CAF-I were analysed and compared separately using a *t*-test analysis. No significant differences were found. The ROUT and Shapiro Wilk methods were used to identify outliers (Q = 1) and study the normality respectively.

3.3. Intestinal enterohormone gene expression is not affected by the different diets in females

We then analysed the expression of genes encoding key enterohormones in females, as alterations in secretion and food intake have been observed in this group. CCK expression in the duodenum remained unchanged, which is consistent with the absence of differences in basal CCK secretion previously reported (Table 5). We then examined GLP-1 expression in the ileum, which also showed no significant variation across treatments due to insect treatment. Surprisingly, beef feeding did lead to a reduction in GLP-1 as well as the enteroendocrine marker ChgA. Finally, we assessed the expression of the ghrelin receptor in the ileum, given earlier findings that suggest chronic modulation by insect feeding. However, under the current experimental conditions, no significant differences were detected.

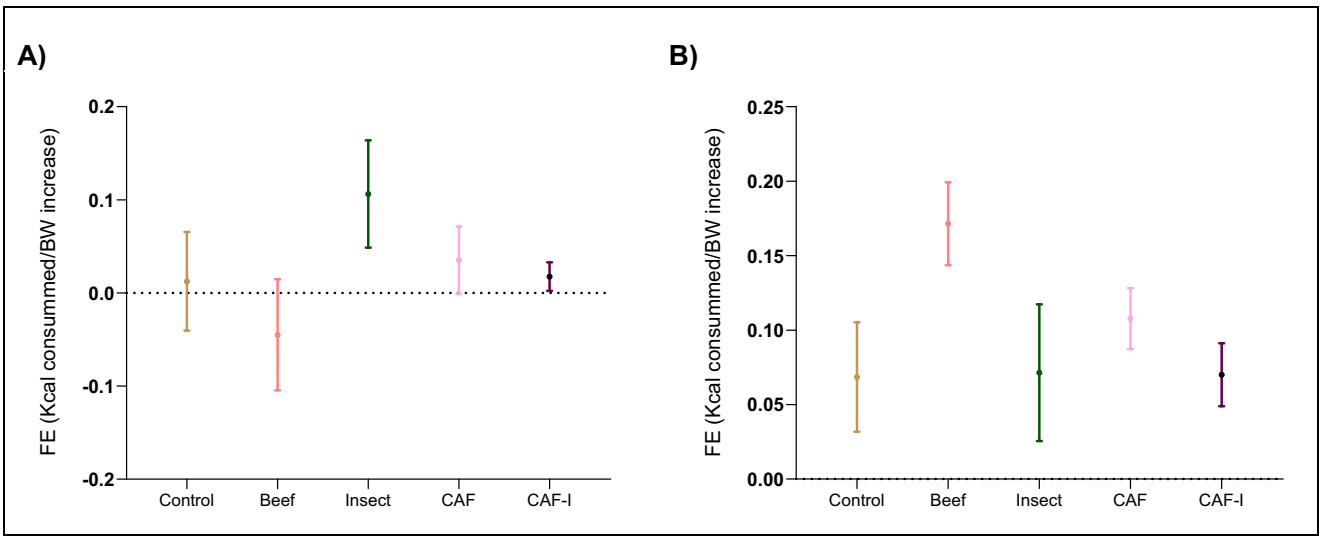

Fig. 4. *Feed Efficiency ratio at week 4 of the experiment (last week before sacrifice). A) Females and B) Males. Results are presented as the mean ± s.e.m. (n = 6–8). Statistics were determined using the One-Way ANOVA Test between standard groups (Control, Beef and Insect groups). CAF and CAF-I were compared separately using a t-test analysis. There were no significant differences between any of the metabolic states, in females or males. The ROUT and Shapiro Wilk methods were used to identify outliers (Q = 1) and study the normality respectively.*

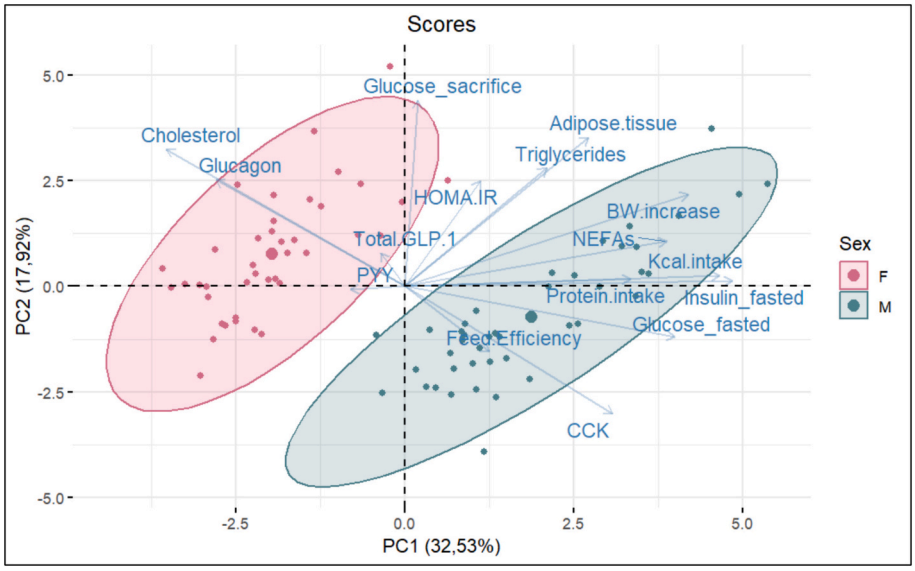

Fig. 5. *Principal Component Analysis (PCA) plot of physiological peripheral variables at the end of the experiment. Each point represents an observation, colored by group. Ellipses show the 95% confidence region for each group. Females are represented in red (F) and Males are in green (M). Arrows indicate the loadings of the original variables, showing their contribution to the first two principal components (PC1 and PC2). The axes display PC1 and PC2, with the percentage of variance explained indicated in parentheses. Variables were standardized prior to PCA to zero mean and unit variance (z-scores), ensuring that all variables contribute equally to the analysis.* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Insects are regarded as an optional source of alternative protein with ecological benefits; therefore, their use as a food and animal feed is being increasingly analysed(Kłobukowski et al., 2025). *A. diaperinus* is one of the insects studied for being added to food (Krawczyk et al., 2024) (Mazurek et al., 2024) and animal feed (Habte-Tsion et al., 2024). We have previously shown that *A. diaperinus* modifies secretome differently than almond and beef, and that it leads to differences in food intake in rats and humans (Miguéns-Gómez et al., 2020). These previous experiments were performed with insect protein administered as a meal load. This mode of protein administration has previously been shown to have different effects than when the sources are administered as protein replacement in food (Miguéns-Gómez et al., 2023). In the present experiment, we tested the effects of protein replacement in food with the peculiarity of conducting this study in both sexes with the aim of delving deeper into the metabolic footprint associated with sex.

Our present study shows in first place that a diet where total protein has been replaced by *A. diaperinus* does not lead to significant body weight modifications or biochemical parameters when compared to casein or beef. This supports using *A. diaperinus* in animal feed as it clearly does not interfere with animal development. This result agrees with those found in Atlantic salmon after a 12-week food replacement diet (Habte-Tsion et al., 2024). Interestingly, in females a reduction in food intake during the last week of the study was observed. This slight reduction did not lead to feed efficiency changes or body weight changes

Table 3
Enterohormones plasma secretion of male and female rats at week 4 of experiment (sacrifice). Results are presented as the mean ± s.e.m. (n = 6–8). One-way ANOVA or Kruskal-Wallis test was used (depending on sample normality) to evaluate the significance between standard groups (P < 0.05). CAF and CAF-I were analysed and compared separately using t-test analysis. # shows a tendency (p < 0.1) between beef and insect group in males. The effect of cafeteria diet was analysed using a t-test analysis versus standard, and no significant differences were found. The Grubbs method was used to identify outliers (Alpha = 0.05) and the Shapiro-Wilk test was performed to analyse normality.

	Females					Males				
	Control	Beef	Insect	CAF	CAF- I	Control	Beef	Insect	CAF	CAF-I
CCK (ng/mL)	0.22 ± 0.03	0.27 ± 0.07	0.38 ± 0.09	0.19 ± 0.02	0.22 ± 0.06	4.08 ± 0.31	3.26 ± 0.47	2.96 ± 0.27	2.64 ± 0.54	1.97 ± 0.36
Active Ghrelin (pg/mL)	54.16 ± 13.67	19.62 ± 6.60	23.02 ± 5.00	27.56 ± 4.68	23.11 ± 13.42	356.63 ± 60.71	209.05 ± 11.46#	459.95 ± 81.69	397.16 ± 64.67	353.57 ± 60.29
Total GLP-1 (pM)	28.22 ± 5.50	25.86 ± 4.65	26.6 ± 4.60	33.65 ± 7.98	32.97 ± 4.11	23.26 ± 1.86	19.14 ± 2.52	15.72 ± 3.29	23.61 ± 2.44	22.63 ± 4.00
PYY (pg/mL)	228.61 ± 17.02	219.50 ± 14.12	248.08 ± 50.20	192.92 ± 6.51	299.50 ± 97.54	71.17 ± 3.39	62.18 ± 3.07	74.59 ± 3.35	96.83 ± 3.54	104.53 ± 4.83

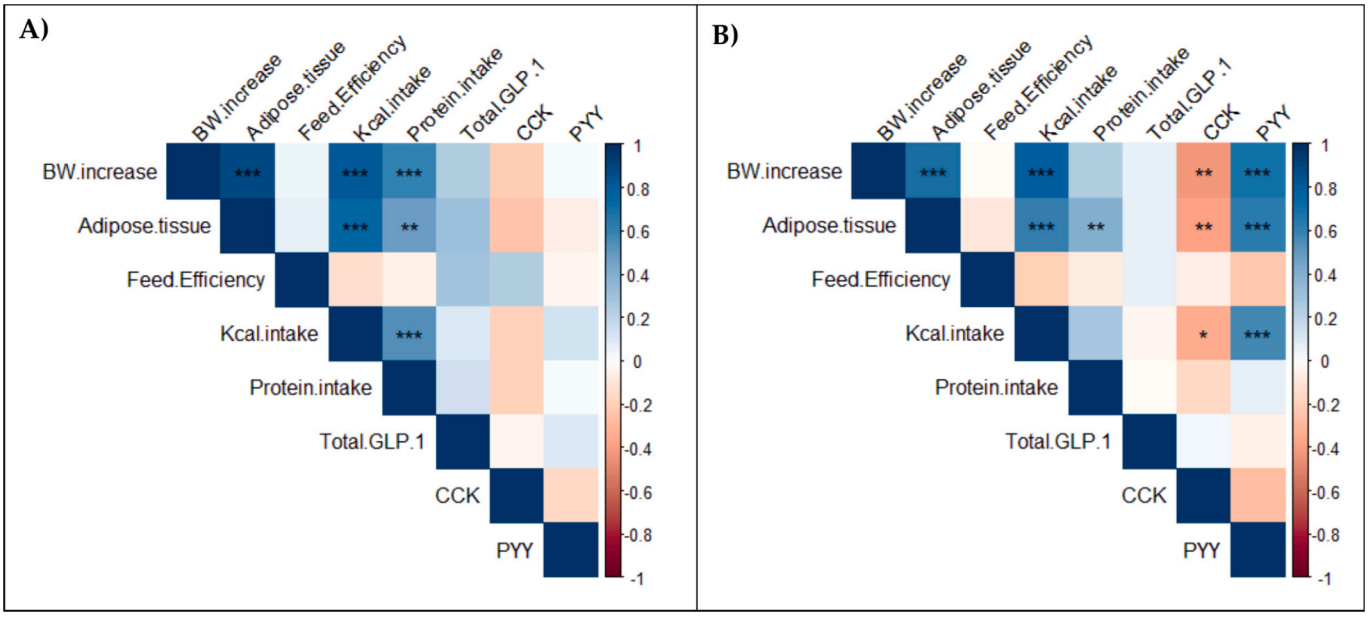

Fig. 6. Heatmap of correlations among physiological parameters at the end of the experiment in females (A) and males (B). Colors indicate correlation strength and direction (blue = positive, red = negative). "Protein intake" refers to the total amount of protein consumed from animal feed, whereas "Kcal intake" represents the total kilocalories ingested, including additional energy derived from the cafeteria diet in the obesogenic groups. Numbers show the correlation coefficients, and asterisks indicate statistical significance: * p < 0.05, ** *p* < 0.01, *** *p* < 0.001. Significance was assessed using a two-sided t-test for Spearman's correlation and *p*-values were adjusted for multiple comparisons using the Bonferroni method. Missing values were imputed using multiple imputation prior to analysis. The Shapiro Wilk methods was used to study sample normality. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at this time point, although more prolonged treatments would be required to determine whether effects on body weight could be observed. The effects on food intake differ from those observed previously when *A. diaperinus* was administered as a meal load, since 300 mg/kg of BW of *A. diaperinus* in female rats increased their food intake. However, this effect only lasted during the initial days of the treatment, and the rats reached a final body weight very similar to that of the control animals (Miguéns-Gómez et al., 2023). In older adult humans, studies have addressed whether the way that protein is administered is important for food and protein intake, although results are inconclusive (Lonnie et al., 2018). Our results thus highlight that when the aim is to modify food intake the mode of administration (as an acute load or as protein replacement) is important.

Our previous results showing a food and body weight intake increase after *A. diaperinus* administration prompted us to include a group of diet-induced obese animals to test the effects of protein replacement. Present results show no clear effect of *A. diaperinus* in these animals. Food intake or body weight were not modified in cafeteria-fed animals due to insect treatment, as it happened in standard diet-fed rats. However, a slight amelioration of lipid profile was observed in *A. diaperinus*-fed animals. Kang et al. have suggested that protein replacement by *A. diaperinus* under an obesogenic diet has beneficial effects in male mice (Kang et al., 2023). In agreement with our results, they did not find differences in food intake or body weight gain. In their experiments triglycerides and total cholesterol where not modified, although the experimental procedures and duration time (8 and 12 weeks) were different from our study.

We previously showed that *A. diaperinus* acutely induces GLP-1 secretion in enteroendocrine cells as well as in jejunum explants (Miguéns-Gómez et al., 2023). Our present results also show that protein replacement by *A. diaperinus* modulates satiating enterohormone secretion. In females, the jejunum was more sensitive than the duodenum, where GLP-1 and PYY secretion were affected. Basal secretion of these enterohormones was increased in the *A. diaperinus* diet compared to the other protein sources. The increase in the intestinal secretion of anorexigenic enterohormones in females agrees with the reduced food

Table 4
Enterohormones intestinal secretion by in vitro experimental (Basal secretion and stimulated with peptone). CCK and Total Ghrelin were measured in the duodenum explants. Total GLP-1 and PYY were measured in the distal jejunum. Results are presented as the mean ± s.e.m. ($n = 6–8$). One-way ANOVA or Kruskal-Wallis test was used (depending on sample normality) to evaluate significance between standard groups ($P < 0.05$). CAF and CAF-I were analysed and compared separately using *t*-test analysis. The Grubbs method was used to identify outliers (Alpha = 0.05) and the Shapiro-Wilk test was performed to analyse normality. NA are not available data. Different letters indicate statistical differences between diets (standard groups).

		Basal secretion					Stimulated secretion				
		Control	Beef	Insect	CAF	CAF + I	Control	Beef	Insect	CAF	CAF + I
Females	CCK (ng/mL)	0.33 ± 0.01	0.32 ± 0.04	0.30 ± 0.03	0.33 ± 0.04	0.29 ± 0.04	0.49 ± 0.11	0.53 ± 0.16	0.36 ± 0.04	0.38 ± 0.06	0.36 ± 0.04
	Total Ghrelin (mg/mL)	0.23 ± 0.35	0.23 ± 0.31	0.36 ± 0.67	0.20 ± 0.43	0.15 ± 0.36	NA	NA	NA	NA	NA
	Total GLP-1 (pM)	8.12 ± 1.71**ab**	4.43 ± 0.60**a**	15.19 ± 3.01**b**	5.33 ± 1.03	4.78 ± 0.88	18.04 ± 4.02	7.89 ± 2.66	22.52 ± 5.80	23.22 ± 4.19	17.13 ± 5.76
	PYY (pg/mL)	0.95 ± 0.36	0.94 ± 0.77	1.53 ± 0.72	2.43 ± 1.97	1.05 ± 0.79	NA	NA	NA	NA	NA
Males	CCK (ng/mL)	0.01 ± 0.00	0.01 ± 0.00	0.12 ± 0.05	0.08 ± 0.05	0.04 ± 0.65	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.03	0.15 ± 0.06	0.07 ± 0.03
	Total Ghrelin (mg/mL)	0.28 ± 0.07	0.28 ± 0.06	0.32 ± 0.08	0.17 ± 0.04	0.17 ± 0.03	NA	NA	NA	NA	NA
	Total GLP-1 (pM)	16.22 ± 4.27**a**	8.08 ± 2.07**ab**	1.86 ± 0.38**b**	3.69 ± 0.95	3.30 ± 0.78	NA	NA	NA	NA	NA
	PYY (pg/mL)	3.78 ± 2.67	4.15 ± 0.91	1.73 ± 0.44	2.55 ± 0.57	2.25 ± 0.47	17.12 ± 0.82	11.62 ± 0.80	12.87 ± 1.48	11.47 ± 1.59	13.87 ± 1.89

Table 5
Enterohormones gene expression in females. CCK was analysed in the duodenum and GLP-1, PYY, ChgA and Ghsr in the ileum. Results are presented as the mean ± s.e.m. (n = 6–8). One-way ANOVA or Kruskal-Wallis test was used (depending on sample normality) to evaluate significance between standard groups ($P < 0.05$). CAF and CAF-I were analysed and compared separately using t-test analysis. The Grubbs method was used to identify outliers (Alpha = 0.05) and the Shapiro-Wilk test was performed to analyse normality. Different letters indicate statistical differences between diets (standard groups).

	Control	Beef	Insect	CAF	CAF- I
CCK	1.19 ± 0.22	1.34 ± 0.12	1.20 ± 0.23	1.16 ± 0.27	1.06 ± 0.17
Ghsr	1.33 ± 0.34	0.88 ± 0.28	0.62 ± 0.09	1.72 ± 0.28	1.33 ± 0.24
GLP-1	1.13 ± 0.21**a**	0.38 ± 0.06**b**	0.79 ± 0.13**a**	0.81 ± 0.13	1.71 ± 0.65
ChgA	1.19 ± 0.26 **a**	0.45 ± 0.09 **b**	0.66 ± 0.10 **a**	0.76 ± 0.14	1.21 ± 0.38
PYY	0.85 ± 0.07	0.61 ± 0.13	0.97 ± 0.18	0.82 ± 0.12	0.68 ± 0.09

intake observed only at the end of the study. Moreover, in cafeteria-fed animals, in which we observed no effects on food intake, basal jejunal secretion was also not modified. Gene expression results in ileum, showing a lack of effects due to insect feeding of GLP-1, PYY, and enteroendocrine marker ChgA, suggest that the GLP-1 basal increased secretion is not due to an increase in the amount of enteroendocrine cells; therefore, promotion of enteroendocrine differentiation does not seem to be the mechanism that explains it. The implications of these basal increasing effects should be further explored, although basal enterohormone secretion is not well studied. However, enterohormone release after stimulation is a well-defined mechanism for modulating the regulation of food intake and glucose homeostasis at the local, central and peripheral modes (Xie et al., 2020). In our experiments we found no effects on stimulated secretion, in agreement with the lack of effects on glucose homeostasis (no changes in HOMA-IR were found). Furthermore, a reduction in ghrelin secretion has been suggested to be involved in the effects of *A. diaperinus* in food intake, and gene expression changes in the ghrelin receptor in the ileum could be the cause of the loss of the effects along time (Miguéns-Gómez et al., 2023). In the present study, we found no indications of ghrelin modification by *A. diaperinus* in the plasma at sacrifice or in the basal secretion from the duodenal samples. Moreover, no changes in duodenal ghrelin secretion were found. Therefore, the route of protein administration plays a crucial role in regulating, and consequently in controlling food intake.

Finally, an important finding of the present study is that the effects of *A. diaperinus* in diet protein replacement are sex dependent. While the previous discussion refers to effects in females, our data shows a completely different profile in males: no effects on food intake, a significantly lower basal GLP1 secretion under insect diet and a trend towards lower basal PYY. In a human study intestinal basal secretion of PYY and GLP-1 did not show sex differences (Jones et al., 2023), and in rats no differences in intestinal gene expression of GLP-1 have been observed (Börchers & Skibicka, 2025). However, a recent revision of animal and human studies on GLP-1 analogues concludes that there is no clear evidence for large qualitative sex differences in the therapeutic effect of these drugs, but there might be quantitative sex differences in how males and females respond to GLP-1 and its analogues (Börchers & Skibicka, 2025). Interaction with oestrogens might play a role in these observations (Börchers & Skibicka, 2025). In addition, sex differences in the expression of nuclear receptors that target genes regulating GLP-1 have been described (Dean et al., 2021). Moreover, there is a sex difference in the ability of GLP-1-producing neurons activation to control motivated behaviour for food (Lopez-Ferreras et al., 2023). Intestinal PYY secretion is also regulated in a sex-specific manner by RET tyrosine kinase (Shepherd et al., 2023). In this line, we found that correlation between enterohormones and physiological parameters are stronger in males than in females. Thus, sexual dimorphism in intracellular signalling, including oestrogen signalling, could be involved in the differential effects of alternative protein feeding on enterohormone release.

Taken together, our results show that the effects of insect protein on food intake depend on the mode of administration (load vs protein replacement in food). Furthermore, we showed that the effects of protein replacement with *A. diaperinus* are sex dependent. In females a healthier plasma profile was found, and intestinal increase in anorexigenic enterohormones is accompanied by a reduction in food intake after four weeks of treatment, although these effects are lost in obesogenic diets. In males, also the results show that *A. diaperinus* is a suitable source of protein that does not lead to any modifications in body weight or food intake.

Abbreviations

CCK	cholecystokinin
GLP-1	glucagon-like peptide 1

(*continued on next page*)

(*continued*)

PYY	peptide YY
BSA	bovine serum albumin
BW	body weight
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
Ghsr	Growth Hormone Secretagogue Receptor
ChgA	Chromogranin A
SEM	standard error of the mean

CRediT authorship contribution statement

Oria Soler: Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **Mònica Lores:** Writing – review & editing, Methodology, Investigation. **Esther Rodríguez-Gallego:** Writing – review & editing, Supervision, Funding acquisition. **Ximena Terra:** Writing – review & editing, Resources. **Raúl Beltrán-Debón:** Writing – review & editing, Resources, Formal analysis. **Albert Ribas-Agustí:** Writing – review & editing, Methodology, Investigation. **Grau Matas:** Methodology, Investigation. **Anna Ardévol:** Writing – review & editing, Investigation, Conceptualization. **Montserrat Pinent:** Writing – original draft, Supervision, Project administration, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2026.118423.

Data availability

Data will be made available on request.

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