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Identification of animal species housed and herding practices in ancient sediments from the Vallone Inferno rock-shelter (Scillato, Sicily, Italy) using faecal biomarkers, hormones, and their metabolites

Asier Vallejo [a,b,*], Vincenza Forgia [c], Josep Maria Vergès [d,e], Ane Gorostizu-Orkaiztegi [a,b], Amaia Alday-Izaguirre [a,b], Ainhoa Elejaga-Jimeno [a,b], M. Carmen Sampedro [f], Alicia Sánchez-Ortega [f], Ramón J. Barrio [a,b]

[a] Department of Analytical Chemistry, University of the Basque Country (UPV/EHU), Faculty of Pharmacy, Paseo de la Universidad 7, 01006, Vitoria-Gasteiz, Spain
[b] Bioaraba, Metabolómica Biosanitaria, Alimentaria y Medioambiental, Plaza del Renacimiento 6, 01007, Vitoria-Gasteiz, Spain
[c] Dipartimento Culture e Società, Università di Palermo, Viale delle Scienze - Ed.15, 90128, Palermo, Italy
[d] Institut Català de Paleoecologia Humana i Evolució Social (IPHES-CERCA), Zona Educacional 4, Campus Sescelades (Edifici W3), 43007, Tarragona, Spain
[e] Universitat Rovira i Virgili, Departament d'Història i Història de l'Art, Avinguda de Catalunya 35, 43002, Tarragona, Spain
[f] Central Service of Analysis (SGiker), University of the Basque Country (UPV/EHU), Laskaray Ikergunea, Miguel de Unamuno 3, 01006, Vitoria-Gasteiz, Spain

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ABSTRACT

The interest in the identification of animal species housed in caves or rock-shelters used as livestock pen and herding management along prehistoric and historic ages, is increasing to understand better the development of pastoral activities. In this manuscript, a method for the quantification of β-sterol/phytosterols, bile acids, hormones and hormones metabolites has been developed to determine the main pastoral activities carried out in Vallone Inferno rock-shelter (Scillato, Sicily, Italy) from Middle Neolithic to Early Middle Age. According to the result obtained, the main animals housed in the rock-shelter went gradually changing from ovicaprids in Middle Neolithic to pigs in Early Middle Age. Additionally, new proxies (progesterone/Σbile acids and metabolites of progesterone/Σbile acids) were used to detect a high hormonal activity at Early Middle Age samples related with female pig management.

1. Introduction

The introduction of domesticated animals during the Neolithic was the beginning of a complex web of new livelihood strategies in Europe. Domestication of herd animals facilitated the expansion of farming and ensured milk production for caloric security of pioneering farmers (McClure, 2015). Moreover, pastoral practices are resilient to environmental constrains, through the adaptation to various environments, the exploitations of a wide range of resources and the modification of the strategies in the management itself of the herd animals (Brochier et al., 1992; McClure, 2015; Martín et al., 2023).

The use of caves and rock-shelters as livestock pen has been a common practice in animal husbandry from the beginning of the domestication, along the recent prehistory to the historical periods, with some modern and contemporary evidences (Fernández-Eraso and Polo Díaz, 2008; Oms et al., 2008; Angelucci et al., 2009; Polo-Díaz and Fernández-Eraso, 2010; Vergès et al., 2016a, 2016b; Forgia et al., 2021). One archaeological evidence for such use, localized mainly in the entrance areas of those natural shelters, are sediments called *fumiers* deposits. These deposits are composed of alteration of burnt or unburnt animal dung and vegetal remains which are commonly interpreted as the product of pastoral activities. These activities were mainly related with the overnight stay of the herd and the periodic burning of the waste for the cleaning of the pen (Angelucci et al., 2009). These sediments usually consist of different layers or *facies* with different polychromes depending on the thermal alteration: burnt layer (ash white) on the top, partially burnt layer (brownish-grey), and unburned layer (organic matter layer, black colour) on the bottom (Gea et al., 2017). These deposits are commonly found from the Neolithic to the Bronze Age. Later, the pastoral activity can be continued in the site, but these deposits disappear.

* Corresponding author. Department of Analytical Chemistry, University of the Basque Country (UPV/EHU), Faculty of Pharmacy, Paseo de la Universidad 7, 01006, Vitoria-Gasteiz, Spain.
E-mail address: asier.vallejo@ehu.eus (A. Vallejo).

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Recently, a new archaeological site (a rock-shelter) with an impressive sequence of prehistoric layers of burnt and unburnt *fumier* deposits and a potential unburnt historic pastoral deposit coupled with the remains of the daily domestic activities carried out by pastoral communities, has been identified in Sicily, on the northern part of the island (Forgia et al., 2013, 2021).

The Vallone Inferno rock-shelter is located on the foothills of the Madonie Mountain range, Scillato (Palermo), Italy (Fig. 1) (37° 52′ 17.74″ N and 13°55′ 58.97″ E), at 770 m above sea level, visually dominating the final stretch of the Imera river valley and the coast of Palermo. Vallone Inferno (also known as Fosso Inferno) is a deep gorge where the Inferno stream, in coincidence with this rock-shelter (Vallone Inferno), cut an abrupt section of 5-m archaeological deposit under excavation since 2008 on an area of 30 m^2. The rock-shelter is more than 10 m long and 6 m deep and is oriented to north. The results of the archaeological and paleoenvironmental research and the absolute chronology of the main stratigraphic units allowed the identification of the site as sheepfold during the Middle Neolithic period (end of 6th millennium cal BCE), the last stages of the Sicilian Copper Age and the Early Bronze Age (end 3rd – half 2nd millennium cal BCE), and the Early Middle Age (between the 7th and 9th century cal CE) (Forgia et al., 2013, 2021). To date, four archaeological complexes have been identified, among which complex 3 has provided most of the archaeological remains. A persistent use of the shelter for pastoral activities during prehistory and historic times was suggested from the results of the study of macrofaunal, plant remains, and from the diverse layers of *fumier* deposits: pigs are the basis of historic exploitations, while ovicaprids are the most important taxon in prehistoric times (Forgia et al., 2013, 2021; Martín et al., 2023). The presence of shoots, probably harvested in early spring, and of abundance of deciduous teeth from immature ovicaprine individuals indicate the development of breeding activity in the rock-shelter. Hunting activity has also been documented throughout the sequence, with the presence of a variety of wild mammal species.

The accumulation of dung along the use of sites, like the Vallone Inferno, as livestock pen leaves a trail of faecal biomarkers which can be used to determine the animal species housed in the cave or rock-shelter (Martín, 2015; Martín et al., 2016; Polo-Díaz et al., 2016). Biomarker studies, together with zooarchaeological and micromorphological studies, are useful to ensure that the livestock was enclosure in the site (Vallejo et al., 2022a). Furthermore, these biomarker studies help in the narration of modalities of management of herd along articulated sequences, like the one in the Vallone Inferno rock-shelter, where different husbandry strategies have been recognized, since the early stages of the development of the pastoral economy (end VII - beginning II millennium BCE) until the historical exploitation of the site, during Early Medieval period.

Faecal biomarkers such as β-sterols (coprostanol [Cop], epicopostranol [EpiCop]), β-phytosterols (5β-stigmastanol [5β-Stig] and epi-5β-stigmastanol [Epi-5β-Stig]) and bile acids (ursodeoxycholic acid [Urso], hyodeoxycholic acid [Hyo], chenodeoxycholic acid [Cheno], deoxycholic acid [Deox], and lithocholic acid [Lit]) are the characteristic biomarkers used to classify species, although recently, Harrault et al. (2019), proposed the necessity to increase the number of 5β-sterol and 5β-phytosterols to identify the specie of animal more accurately (Shah et al., 2007; Tyagi et al., 2009; Shillito et al., 2011; Mackay et al., 2020). Hyo and Urso are the unique specific biomarkers of an animal specie, the pig. The identification of the rest of the species needs the use of proxies based on faecal biomarkers concentrations obtained from contemporary samples. (Bull et al., 2002; Gea et al., 2017; Prost et al., 2017; Harrault et al., 2019; Vázquez et al., 2021). The use of these proxies is, until now, the best approach to identify animal species using faecal biomarkers (Table 1) but the uncertainty about their stability along time could be a problem.

Recently, other biomarkers such as hormones (progesterone [PG]

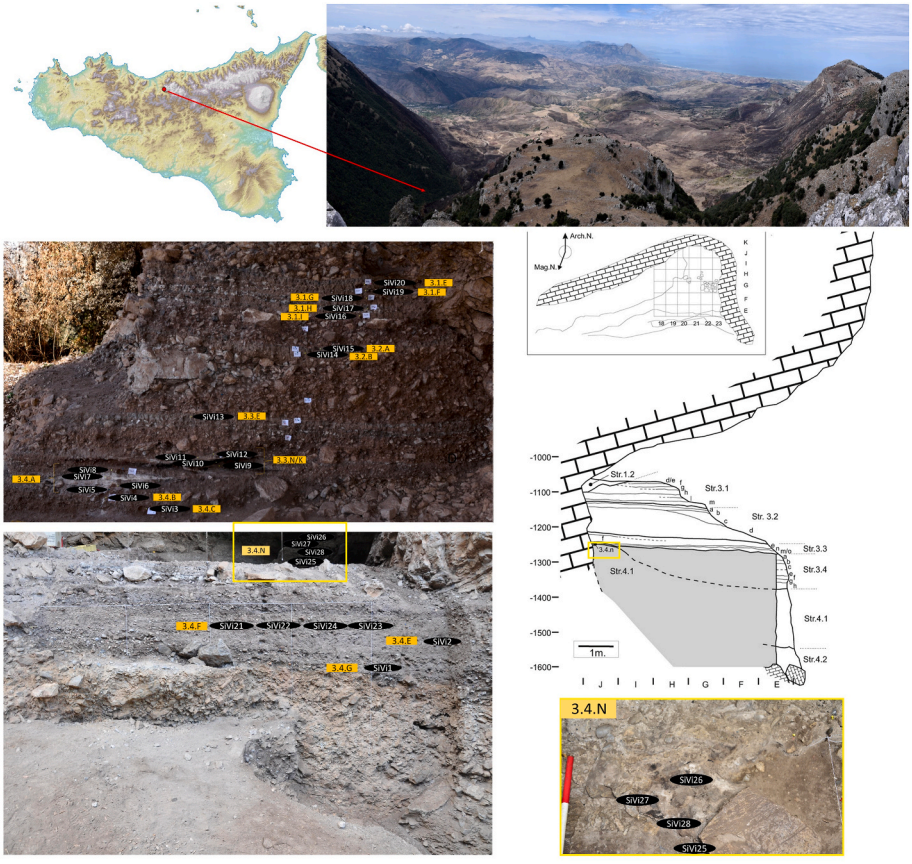

Fig. 1. Vallone Inferno rock-shelter location, levels sample locations, and synthetic stratigraphic sequence.

Table 1
Commonly used proxies from literature to identify animals from faecal remains.

Proxy	Outcome						Reference
$\frac{5\beta - coprostanol + epi - 5\beta - coprostanol}{5\beta - stigmastanol + epi - 5\beta - stigmastanol}$	Omnivore >1	Cholesterol dominant – Canine Coprostanol dominant – Human or pig					Bull et al. (2002)
	Ruminant <1						
Presence of Lit, Deox, Hyo and Hyocholic	Hyo and Hyocholic dominant - Porcine						
	Lit, Deox	Lit ~ Deox - Human Deox dominant - Ruminant					
$\frac{5\beta - coprostanol}{5\beta - coprostanol + 5\beta - stigmastanol} x100$	Herbivore <38%						Leeming et al. (1996)
	Values between 38% and 73% need a 2.86 factor						
	Human >73%						
	Herbivore <29%	$\frac{epi - \beta - stigmastanol}{\beta - stigmastanol} + \frac{epicoprostanol}{coprostanol}$	Horse >1.2	Deox ≥ Cheno Deox ≥ Lit Lit < Cheno			Prost et al. (2017)
			Donkey, cattle, sheep, goat, goose <0.8	Cheno Yes	Goat	Deox » Cheno Deox > Lit Lit ~ Cheno	
					Goose	Cheno » Deox Lit > Deox Cheno > Lit	
				Cheno No	Sheep and Cattle Donkey	Deox > Lit Deox ~ Lit	
	Values between 29% and 65% + Hyo Human >65%	Hyo » Deox Lit » Deox With Urso Deox » Cheno Deox > Lit Lit » Cheno					
$\frac{Deoxycholic\ acid}{Lithocholic\ acid}$	Ruminant >10						Gea et al. (2017)

and testosterone [TT]) have been used to explain prehistoric herding practices (Vallejo et al., 2022a). The ratio PG/Deox was used to determine in El Mirador cave (Sierra de Atapuerca, Burgos) that the pregnant females and their offspring were stabled in the pen and separated from the rest of the herd. PG hormone is excreted by faeces and urine and its concentration varies depending on the menstrual cycle and pregnancy (Liu et al., 2012; Croxatto, 2017). However, PG is partially metabolised into 5α and 5β pregnanes prior to excretion and the concentration of these metabolites is higher in faeces than PG concentration. Therefore, PG metabolites such as Pregnanolone (5βPT1) and 5β-pregnane-3α, 20β-diol (5βPTOH) could be also accumulated in ancient sediments and used as biomarkers of herding practices. TT is more related with males and is also metabolised. One of those metabolites is Androsterone (inactive metabolite) and could be used also to predict herding practices related to males (Schänzer, 1996). Oestrogens, oestrone (E1) and 17β-oestradiol can also be used to predict pregnancy, however, in the first approach made in ancient sediments from El Mirador cave these hormones were no detected (Vallejo et al., 2022a).

The simultaneous analysis and quantification of β-sterols, β-phytosterols and bile acids was already developed by different authors (Bull et al., 2003; Shillito et al., 2011; Birk et al., 2012; Lauer et al., 2014; Gea et al., 2017; Prost et al., 2017; Zocatelli et al., 2017). All these methods combine a total lipid extraction step follows by a hydrolysis (saponification) step, a clean-up steps and a derivatization step before the analysis by gas chromatography-mass spectrometry. Alternately, the analysis can be performed by liquid chromatography-mass spectrometry (LC-MS) to eliminate the derivatization step.

Therefore, the objectives of this manuscript are i) to develop a simultaneous method for the quantification of three β-sterols (Cop, EpiCop and Col), two β-phytosterols (5β-Stig and Epi-5β-Stig), five bile acids (Hyo, Urso, Cheno, Deox, Lit), four hormones (E1, E2, PG, TT) and their metabolites (5Bpt1, 5βPTOH and Andro) using LC-MS to characterise the biogenic origin of ancient sediments from Vallone Inferno rock-shelter (Scillato, Sicily, Italy), ii) determine the l use of the rock-shelter from Middle Neolithic to Early Middle Age and iii) identify any herd distribution according to hormones and metabolites concentration.

2. Material and methods

2.1. Stratigraphy, sampling, storage, and homogenisation of the samples

Twenty-four sediment samples were collected from the accumulated vertical layers from the Early Middle Ages to the last stages of the local Copper Age (Fig. 1 and Table 2).

The archaeological units were established according to sedimentary criteria (Forgia, in press; Forgia et al., 2013). Numbers were used to designate complexes and sub-complexes; letters were used to refer to individual stratigraphic units. The latter were specified based on small sedimentary differences or archaeological features, such as the presence of burnt sediments, structures, etc. From the four main stratigraphic complexes identified, complex 3, i.e. the archaeological succession, was the object of our study. The complex consists of an alternation of fine-textured archaeological layers and coarser beds. In subcomplex 3.4, the earliest archaeological record (unit 3.4.n partially preserved and dated to 5380-5210 BCE) is followed by a phase of erosion and then by a new phase of sedimentation (units 3.4.a-h), between the last stages of the Sicilian Copper Age and the Early Bronze Age (end 3rd – half 2nd millennium cal BCE). After a gap, a rapid sedimentation occurred between 7th and 9th century CE (subcomplexes 3.1–3.3). The detailed description of each stratigraphic unit is in the monographic volume by

Table 2
Samples collected from Vallone Inferno rock-shelter (Scillato, Sicily, Italy). Samples name, level, data (cal 2sigma. 95%, Forgia et al., 2013, 2021), and facies information is described based on (Angelucci et al., 2009; Vergès et al., 2022).

Samples	Level	Data (cal 2sigma. 95%) (Forgia et al., 2013, 2021)	Facies info.	Description (Angelucci et al., 2009; Vergès et al., 2022)
SiVi20	3.1.E		Dung	
SiVi19	3.1.F			
SiVi18	3.1.G			
SiVi17	3.1.H			
SiVi16	3.1.I	665-875 CE		
SiVi15	3.2.A		Dung	
SiVi14	3.2.B	650-775 CE		
SiVi13	3.3.E		Dung	
SiVi12	3.3. N/K		Fumier deposit grey layer (g)	Light grey silt, massive, with abundant ash dispersed in the matrix
SiVi11	3.3. N/K		Fumier deposit white layer (w)	Accumulations of ash, almost pure; sometimes contains charcoal fragments or yellowish small mottles; occasionally shows fine parallel lamination
SiVi10	3.3. N/K		Fumier deposit charcoal layer (c)	Accumulations of mm-to cm- sized vegetal charcoal fragments
SiVi9	3.3. N/K		Fumier deposit grey layer (g)	
SiVi8	3.4.A		Fumier deposit A layer (A)	Yellowish brown clayey silt, with few to common unsorted calcareous stones, common organic matter, high porosity; it contains common ash and scarce microcharcoal fragments dispersed in the matrix
SiVi7	3.4.A		Fumier deposit white layer (w)	
SiVi6	3.4.A		Fumier deposit tutti frutti layer (tf)	Silt with abundant ash and varied colors, sometimes with platy structure and moderate cementation
SiVi5	3.4.A		Fumier deposit charcoal (c)	
SiVi4	3.4.B	1620-1420 BCE		
SiVi3	3.4.C			
SiVi2	3.4.E			
SiVi21	3.4.F		Roasted dung	
SiVi22	3.4.F		Roasted dung	
SiVi23	3.4.F		Roasted dung	
SiVi24	3.4.F		Roasted dung	
SiVi1	3.4.G	2570-2300 BCE		
SiVi26	3.4.N		Fumier deposit white layer (w)	
SiVi27	3.4.N	5380-5210BCE	Fumier deposit charcoal layer (c)	
SiVi28	3.4.N		Fumier deposit grey layer (g)	
SiVi25	3.4.N			

Forgia and colleagues (Forgia, in press).

The facies were collected with a steal stainless spoon covered with aluminium foil to avoid contamination. The aluminium foil was removed ones the sample was collected. All samples were stored in aluminium foil and transported in zip-bags at 4 °C. Once in the laboratory, the samples were freeze and dried, milled, sieved to 60 μm particle size fraction, and stored at −82 °C until analysis.

A deeper description of the facies type used in this manuscript was done by Angelucci et al. and Vergès et al. (Angelucci et al., 2009; Vergès et al., 2022)

2.2. Chemical, reagents, and reference standards

Sterols (epicoprostanol [Epicop, 95%], copostranol [Cop, 98%] and cholestanol [Col, 94.8%]), bile acids (ursodeoxycholic acid [Urso, 99%], hyodeoxycholic acid [Hyo, 99%], chenodeoxycholic acid [Cheno, 97%], deoxycholic acid [Deox, 98%], and lithocholic acid [Lit, 95%]), hormones (17β-oestradiol [E2, 99.7%], oestrone [E1, 99%], progesterone [PG, 99.6%] and testosterone [TT, 99.9%]), hormones metabolites (pregnanolone [5βPT1, >98%], 5β-pregnane-3α,20β-diol [5βPTOH, >98%], androsterone [Andro, 98.2%]), and three isotopically labelled compounds (17β-oestradiol ([$^2H_3$]-E2, 98%), lithocholic acid ([$^2H_5$]-Lit, 98%) and cholesterol ([$^2H_7$]-CHOL, 98%) were obtained from Sigma-Aldrich (Madrid, Spain).

Phytosterols (epi-5β-stigmastanol [Epi-5β-Stig, 99%] and 5β-stigmastanol [5β-Stig, 99]) were supplied by Chiron (Trondheim, Norway) and deuterated β-sitosterol ([$^2H_7$]-Sit, 98%) by C/D/N Isotopes (Point Claire, Quebec, Canada).

Dichloromethane (DCM, HPLC grade), methanol (MeOH, HPLC grade), and potassium hydroxide (KOH) were purchased from Scharlab (Barcelona, Spain), and hydrochloric acid (HCl) from Fisher Scientific (Loughborough, UK).

Ultra-high purity water used for mobile phase, saponification and cleaning step was obtained from tap water that was pre-treated by Elix reverse osmosis and subsequent filtration using a Milli-Q system from Millipore (Bedford, MA, USA).

2.3. Homogenised samples workflow

Homogenised samples workflow was performed based on Vallejo et al. (2022a) optimised method. Briefly, 5 g of homogenised sample was extracted by a microwave oven using 25 mL of DCM:MeOH (2:1 v/v) mixture. Afterwards, the extract was centrifugated and the supernatant was evaporated to dryness before the saponification step. Next, the solution was acidified using 2 M HCl/H_2O ($pH = 2 \leq x \leq 4$). Finally, a continuous liquid-liquid extraction was carried out with DCM (2×10 mL), the organic phase was collected, evaporated to dryness, recovered in MeOH, and after a centrifugation, the supernatant was transferred to chromatography vial inserts.

2.4. Instrumental analysis

Agilent 1290 infinity liquid chromatography system (Palo Alto, SA, USA) was used for the analysis of the samples and Ascentis Express C_{18} HPLC column (15 cm, 3.0 mm, 2.7 μm; Supelco, Sigma-Aldrich) was used for chromatographic separation. The injection volume was 1 μL,

the mobile phase flow rate was set to 0.6 mL/min, and the temperature to 35 °C. The mobile phase gradient was as follows (Solvent A was Milli-Q water, and solvent B was MeOH): 0 min 35% A and 65% B; 3 min 26.3% A and 73.7% B; 4 min 25.0% A and 75.0% B; 8 min 15.0% A and 85.0% B; 10 min 1.6% A and 98.4% B; 20 min 0.9% A and 99.1% B; 22 min 0.0% A and 100% B; 23 min 35.0% A and 65.0% B.

A mass spectrometer (Agilent 6120) was used for atmospheric pressure chemical ionisation (APCI). The scan event was conducted in the positive-ion mode. The source conditions were set as: capillary voltage, 3000 V; drying gas temperature, 300ºC; drying gas flow, 3.0 L/min; nebuliser gas pressure, 20 psig. Measurements were performed in the selected ion monitoring (SIM) mode. The first ion was used as the quantifier and the second as the qualifier for all compounds (E2: 255.2/271.0; E1: 271.1/253.1, [$^2H_3$]-E2: 258.2/249.1; TT: 289.2/271.1; Urso: 357.2/358.2; PG: 315.2/313.1; Andro: 255,2/273.2; Hyo: 357.2/358.2; 5βPT1: 301,2/283; Cheno: 357.2/339.1; Deox: 357.2/355.0; 5βPTOH: 285/301; [$^2H_5$]-Lit: 364.3/346.3; Lit: 359.3/341.2; EpiCop: 371.3/372.4; [$^2H_7$]-CHOL: 377.1/393.1; COP: 371.3/385.3; Epi-5βStig: 399.5/400.3; Col: 371.3/383.3; [$^2H_7$]-Sit: 405.1/421.1; 5βStig: 399.4/400.4).

2.5. Quality parameters of the method. Figures of merit

The method used for the determination of the target analytes was based on a previously optimised method by Vallejo et al. (2022a). Nevertheless, the analysis of β-sterols, β-phytosterols and the hormone metabolites was not included in that method so the determination of the figures of merit for all the analytes was redone.

Calibration of the analytes was performed using matrix blanks spiked with a 3-500 ng/g concentration range of each analyte and 75 ng/g of the deuterated analogues. Non-spiked matrix blanks were also analysed to ensure the absence of the analytes in the matrix blanks spiked. Correlation coefficients greater than 0.99 were obtained in all the cases.

The apparent recovery was calculated at two levels $2xC_{LOQ}$ and $10xC_{LOQ}$. Acceptable values were obtained (60-127%) at both levels and repeatability was always below 21% (RSD%). Limits of detection and quantification were at low ng/g levels (Table 3).

2.6. Principal component analysis (PCA)

To illustrate the possible grouping and similarities among the samples (sediments), a principal component analysis (PCA) was performed.

Table 3
Quality Control Parameters of the analytical method. High and low concentration apparent recoveries (%), repeatability (relative standard deviation, RSD [%]), and limits of detection (C_{LOD}, ng/g) and limits of quantification (C_{LOQ}, ng/g) obtained using this methodology.

Analytes	Low conc. recoveries (%)2 x C_{LOQ}	RSD (%)	High conc. recoveries (%) 10 x C_{LOQ}	RSD (%)	C_{LOD} (ng/g)	C_{LOQ} (ng/g)
E_1	72	2	73	8	24	42
E_2	108	15	76	2	2.8	6.0
TT	95	20	102	21	2.4	3.4
Andro	93	21	67	19	3.3	5.1
PG	103	19	77	21	2.3	2.9
5βPT1	92	6	82	4	5.2	7.6
5βPTOH	127	7	119	1	4.9	11.6
Urso	121	8	82	11	3.1	4.5
Hyo	104	16	89	12	5.9	13.9
Cheno	96	17	111	9	4.3	4.5
Deox	80	8	67	7	9.8	18.3
Lit	109	19	70	10	5.1	14.9
EpiCop	87	15	89	3	2.5	6.6
COP	98	18	66	6	4.0	10.3
Col	102	20	86	6	3.8	9.1
Epi-5βStig	126	17	90	9	10.5	15.6
5βStig	98	13	60	10	5.9	8.2

For this purpose, the logarithmic values of the concentrations of the sterols, phytosterols and bile acids were used, and the missing values were filled with $C_{LOD}/2$ values of each compound. These values were centred and normalised.

3. Results and discussion

3.1. Analysis of the quantification of bile acids and sterol/phytosterols

The behaviour of both target analyte families was similar along all the samples (Fig. 2, Table 1S and Fig. 1S). The husbandry activity was continuous in the rock-shelter from Middle Neolithic to Early Middle Ages except in samples SiVi7, SiVi10 and SiVi11 according to the concentrations of the biomarkers. In these samples, the concentrations of the target analytes were below their C_{LOD} except for Deox and Col in SiVi11, however, these samples corresponded to white and charcoal facies of a *fumier* deposit (SiVi 5-6; SiVi 9) were concentrations of biomarkers attest the husbandry activity. The absence of the biomarkers in samples SiVi7 and SiVi11 (facies type b) was already documented in literature (Gea et al., 2017; Vallejo et al., 2022a), which could be related to a main composition of wood ash of some of the sedimentary facies of fumier deposits (Polo-Díaz et al., 2016). In addition, thermal alteration from high combustion temperatures could have contributed also to significantly reduce the biomarker signal of these samples in the case of dung concentration in the samples. At the top of the archaeological sequence (7th – 9th century cal CE - between SiVi13 and SiVi20), as in the rest of the sequence, the archaeological record is very scarce due to the use of the shelter as livestock pen and as place of daily activities of the pastoral groups settled here. The pottery record was represented by types of common use and amphorae for transport and storage. Furthermore, the abundance of remains of fire pottery, even of considerable size, found in association with combustion structures could be linked to the transformation of dairy products and suggests a specific use of the investigated area of the shelter. It is interesting that units showing a poor archaeological record or where the archaeological record was absent (3.3.E, 3.2.A, 3.1.G, 3.1.F and 3.1.E) showed concentration of biomarkers. Probably, this could be attributed to the exclusive use of this area of the shelter as livestock pen. SiVi8 is a sample of the prehistoric sequence, from the top of the unit 3.4.A, dating to the half of the 2nd millennium BCE. A geospatial analysis conducted for the interpretation of the spatial distribution of archaeological remains from this unit (Forgia et al., in prep.) identifies hotspot where concentration of the human activity produced the accumulation of remains of material culture, in the same area of a structured fireplace opposed to areas (excavation squares without statistically significant archaeological material clusters) in which the shelter must have been used as livestock pen. Significantly, it is possible to note how the excavation squares in which clusters of ceramic finds are absent are precisely those contiguous to the burnt dung levels (*fumier*) in the western part of the excavation area, where the SiVi8 sample comes from.

The lack of biomarker signal in sample SiVi10 (facies c) could be explained by the sparse dung input and heterogeneous composition attributed to facies type c as the floor of the penning area before combustion (Polo-Díaz et al., 2016).

Conversely, the accumulation of β-sterols and β-phytosterols in SiVi6 were below the C_{LOD} and in SiVi4-SiVi6, SiVi23 and SiVi24 below β-sterols C_{LOD}. However, all of them showed β-phytosterols and bile acids accumulation. Therefore, faecal input was considered in these samples owing to the source of β-phytosterols, and bile acids are faeces/manure/dung and because these concentrations were above the background concentration (Bull et al., 2002; Vallejo et al., 2022a). Besides, SiVi5 and SiVi6 samples belonged to c and tf facies respectively, from fumier deposits which were related to faecal inputs and husbandry activity (Vergès et al., 2016a). In the rest of the samples, according to biomarkers concentrations, the rock-shelter was also used as livestock pen although *fumier* deposits were not observed (Table 3). The main

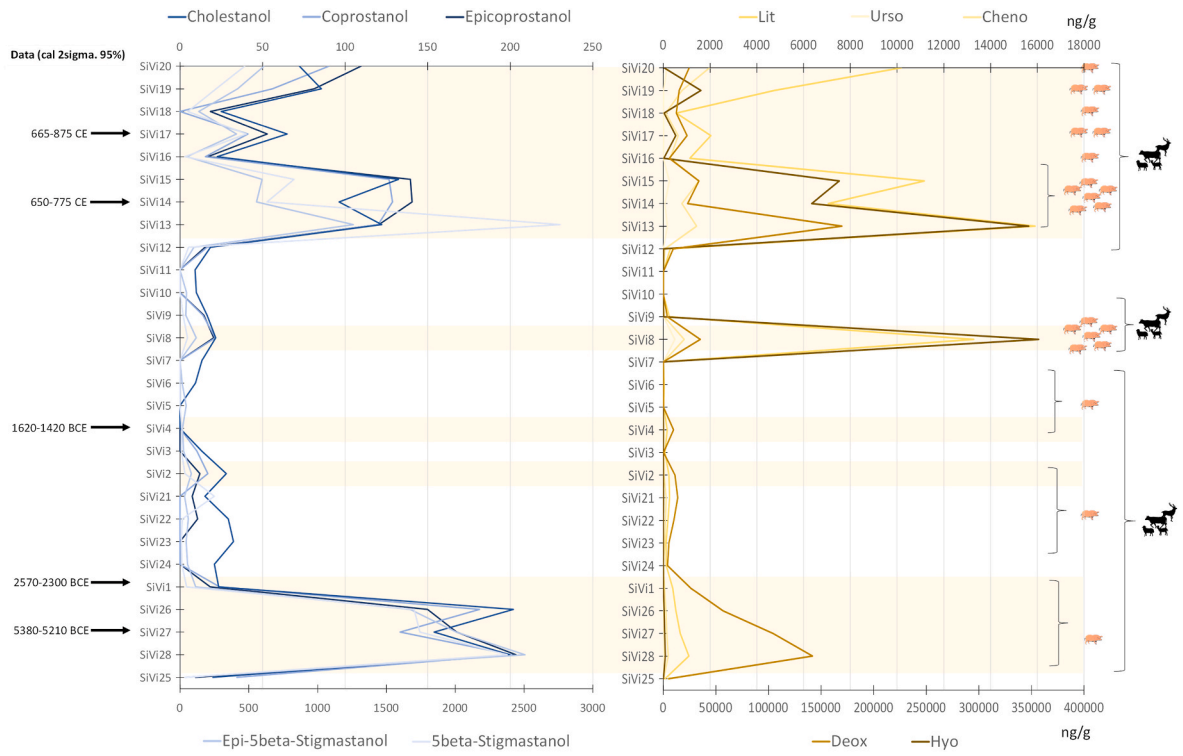

Fig. 2. Concentration (ng/g) of the target analytes and biogenic classification along the samples. Deoxycholic acid, hyocholic acid, epi-5β- and 5β-stigmastanol are illustrated in a secondary axis (below). One pig means low porcine activity, two pigs moderate porcine activity and, six pigs high porcine activity. Highest herding practices in the Vallone Inferno are highlighted in light brown. The samples are ordered from Middle Neolithic Age (on the bottom, after the stratigraphic unit absolutely dated to 5380-5210 cal BCE) to the Early Middle Ages (on the top, 7th – 9th century cal CE). Data is illustrated at the left side.

Table 4
Proxies of stanols, phytostanols and bile acids used for the classification of the samples to determine the housed animals in the Vallone Inferno rock-shelter.

Sample	$\frac{COP}{(COP+5\beta-Stig)} \times 100$ (Prost et al., 2017) (Shah et al., 2007)	$\frac{(EpiCop+Cop)}{(EpiCop+Cop+5\beta-Stig+Epi-5\beta-Stig)} \times 100$	$\frac{(EpiCop+Cop)}{(Epi-5\beta-Stig+5\beta-Stig)}$ (Bull et al., 2002)	$\frac{Deox}{Lit}$	$\frac{Deox}{Cheno}$	$\frac{Cheno}{Lit}$	$\frac{Hyo}{Deox}$	$\frac{Hyo}{Lit}$
SiVi20	16	16	0.18	2.4	12.4	0.2	0.03	0.071
SiVi19	18	17	0.20	3.2	18.8	0.17	2.4	7.5
SiVi18	0	9	0.10	22.3	112.2	0.20	0.056	1.2
SiVi17	8	10	0.11	11.1	35.7	0.31	0.52	5.8
SiVi16	31	27	0.38	5.1	19.5	0.26	0.082	0.4
SiVi15	13	16	0.19	3.0	22.3	0.14	4.9	15
SiVi14	17	18	0.23	3.3	29.1	0.11	6.1	20
SiVi13	4	6	0.06	10.7	119.4	0.09	2.0	22
SiVi12	21	16	0.19	31.8	133.5	0.24	0.025	0.8
SiVi11	0	0	0	No Lit	0	0	0	0
SiVi10	0	0	0	0	0	0	0	0
SiVi9	38	30	0.44	14.8	98.5	0.15	0.061	0.9
SiVi8	28	20	0.24	2.6	39.3	0.07	10.2	26.9
SiVi7	0	0	0	0	0	0	0	0
SiVi6	0	0	0	1.5	0	0	1.0	1.6
SiVi5	0	0	0	10.1	0	0	0.051	0.5
SiVi4	0	0	0	58.4	499.5	0.12	0.004	0.3
SiVi3	51	22	0.29	3.6	15.1	0.24	0	0
SiVi2	32	20	0.24	45.8	382.7	0.12	0.002	0.1
SiVi21	0	3	0.03	51.1	113.0	0.45	0.011	0.6
SiVi22	0	12	0.14	50.6	106.1	0.48	0.003	0.2
SiVi23	0	0	0	44.7	84.0	0.53	0.003	0.1
SiVi24	0	0	0	32.1	0	0	0	0
SiVi1	33	21	0.26	65.0	505.5	0.13	0.004	0.3
SiVi26	16	15	0.18	88.7	446.1	0,20	0.0085	0.76
SiVi27	12	13	0.15	120	617.5	0,19	0.0082	0.98
SiVi28	13	13	0.15	107.5	613.3	0,18	0.0088	0.94
SiVi25	49	49	0.90	64.8	220.6	0,29	0.015	0.96

animal enclosing activity in the Vallone Inferno was detected in SiV8 (facies of a *fumier* located at 3.4.A level), SiVi26-SiVi28 (facies of a *fumier* located at 3.4.N) and, in the samples between SiVi13 and SiVi20 (Fig. 2 and Table S1). In these samples, the concentration of the target analytes increased considerably compared to the rest of the samples. This increase can be related with the number of animals housed in the pen.

Thus, we concluded that the Vallone Inferno rock-shelter was used as livestock pen from Middle Neolithic (after the stratigraphic unit absolutely dated to 5380-5210 cal BCE) to the Early Middle Ages (7th – 9th century cal CE) and the amount of animal heads was changing along the time increasing considerably at Early Middle Ages. Besides, units with poor or absent of archaeological record can be characterized as animal pen using faecal biomarkers even determining the exact point of the pen. Conversely, units with low concentration of biomarkers due to high thermal activity can be classified as animal pen analysing the micromorphology of soils and the archaeological records obtained.

3.2. Biogenic classification of the samples using bile acids and sterol/phytosterols proxies

The biogenic origin of the sample was determinate using different proxies obtained from literature (Table 1) and our own data obtained from the analysis of different species (sheep, goat, cattle, horse, bison, wild pig, deer) feeding in different locations along the North of Spain (Table S2 and Table S3). The own data was obtained analysing 3 individuals from each specie and location. The concentration of Epi-5β-Stig is missing in this data because it was not quantified in that experiment.

According to proxies proposed by Shah eta al. (2007) and Prost et al. (2017), and using sterols and β-phytosterols, SiVi26-SiVi28, SiVi20, SiVi19, SiVi17, SiVi15-SiVi12 and SiVi8 samples corresponded to herbivore residues because the values obtained were below 0.3 (Table 4) (Shah et al., 2007; Prost et al., 2017). SiVi1, SiVi9 and SiVi16 samples values were below 0.38. In these cases, according to Shah et al. (2007), these remains belonged to herbivores while according to Prost et al. (2017) to pigs. Prost et al. (2017) proposed that values between 0.29 and 0.69 and with the presence of Hyo corresponded to pig remains. However, in these cases, if we included the EpiCop and Epi-5β-Stig concentrations in the equation due to their high concentrations in the samples, all the proxies decrease under 0.3. The samples SiVi3 and SiVi25 must be highlighted. In SiVi3, the proxy Cop/(Cop+5β-Stig)x100 value is 51% which according to Prost et al. (2017), the remains belonged to pig, but in this case, the concentration of Hyo was under the limit of detection. However, if we included the EpiCop and Epi-5β-Stig concentrations the value decreased to 20, which belongs to herbivore remains. Thus, although in contemporary samples the concentrations of these isomers are not high enough compared to their partners, the transformation of these isomers along the years is not studied yet, so it is interesting to include them in the proxies in order to keep their robustness (Shah et al., 2007; Prost et al., 2017; Harrault et al., 2019). In the case of SiVi25, the proxy value is 49 and the epimers concentration are below C_{LOD} so there was not any change in the proxy when epimers were included. Therefore, the origin on the sample should be pig, however, the rest of the proxies consider this sample as ruminant origin. Additionally, using the proxy proposed by Bull et al. (2002), all the samples with faecal input belonged to ruminants remains because all the values were below 1 (Table 4).

Conversely, if we include the bile acids in the discussion, the results obtained just with sterols and β-sterols changed. It was clear that the presence of Hyo correspond to porcine remains because this is a specific biomarker excreted just by porcine species (Table S3) (Bull et al., 2002; Prost et al., 2017). Consequently, all the sample except SiVi3, SiVi7, SiVi10, SiVi11 and SiVi24 had porcine remains. The concentration of Hyo in the samples SiVi8, SiVi13-SiVi15 was very high, lower in SiVI19 and SiVi17 and quite low in the rest (Fig. 1 and Table 1S). This suggests a high porcine occupation during the historic phases of exploitation of the rock-shelter, corresponding to the Early Middle Ages (650-875 cal CE) and lower at prehistoric ones, between the Middle Neolithic Age and the Early Bronze Age (mid-end 3rd – mid 2nd millennium BCE). However, the other ratios that confirm the porcine remains did not fit, which suggests a simultaneous animal mixture livestock pen in the rock-shelter. As explained above, the stanols proxies suggested herbivore or ruminant remains. In the case of (COP + EpiCOP)/(5β-Stig + Epi-5β-Stig) proxy, for porcine or humans, the value must be higher than 1 (Bull et al., 2002; Harrault et al., 2019). Nevertheless, Prost et al. (2017) analysed two types of pigs and, using this equation, the values obtained were 0,46 and 0,57. Even in our study, the value of the wild pig was near to one without Epi-5β-Stig (Table 3S) (Prost et al., 2017). This behaviour can suggest that depending on the feeding of the pigs, this value could be below 1 and could explain lower values than one in Vallone Inferno samples. Consequently, for just porcine remains, the concentration of Hyo and Lit must be higher than Deox concentration (Table 3S) (Bull et al., 2002; Prost et al., 2017) and, in Vallone Inferno samples, this behaviour was not observed. The concentration of Hyo was the highest in SiVi19, SiVi15-SiVi13 and in SiVi8, but in any of them Lit concentration was higher than Deox which suggests an input of Deox related to herbivores. In SiVi20, SiVi18-SiVi16, SiVi12, SiVi9, SiVi5-SiVi2, SiVi21-23, and SiVi25-SiVi28 the concentration of Deox was higher or similar to Hyo concentration (Table 3S or (Bull et al., 2002; Prost et al., 2017) which suggests, again, a Deox input related with herbivores. Besides, Hyo concentration should be higher than Lit concentration but in SiVi20, SiVi16, SiVi12, SiVi9, SiVi5-SiVi2, SiVi21-23, and SiVi25-SiVi28 did not happen (Table 4). Again, this was related with a large number of herbivores and small number of pigs enclosed in the rock-shelter because herbivores excreted Lit but not Hyo.

To sum up, the Vallone Inferno was used as livestock pen along almost 5000 years. Other kind of data (pottery and fauna) would agree with the same use of the place as shelter for herds. At prehistoric time, at least between the mid-end of the 6th and the mid of the 2nd millennium cal BCE the rocks-shelter was used as livestock pen mainly for ruminants and few pigs (Martín et al., 2023). This behaviour was changing, and the number of pigs housed was increasing until SiVi8 which corresponds to a period of transition between the final stage of the Early Bronze Age and the beginning of Middle Bronze Age, until being the highest in SiVi19 and SiVi15-SiVi13 which correspond to historic exploitation. The percentages of animal species identified through zooarchaeological studies such as ovicaprids, pigs, cattle and wild species showed a higher presence of pigs in the same levels. This study corroborated the results obtained with the biomarkers (Forgia et al., 2013).

In the case of herbivores, it is difficult to estimate which herbivores were enclosure in the Vallone Inferno due to the animal mixture. According to Prost et al. (2017), the presence of Cheno can be related to horses, goats, or gooses, but just in the case of goat the concentration of Deox was much higher than Cheno concentration (Table 4) so we can conclude that the remains belonged to goats. Nevertheless, in our study, all the herbivores excreted Cheno and just in the cases of sheep and goat the concentration of Deox was much higher than Cheno concentration (Table 3S). Besides, the concentration of Lit was always higher in the Vallone Inferno samples than Cheno and this behaviour just happened in wild pig, sheep, and goat faeces (Table 3S). Besides, the high amount of Lit could be explained due to the presence of pig in the rock-shelter. Thus, we can conclude that goats or sheep were also enclosed in the rock-shelter, but this last affirmation must be taken with care because these species have not a specific biomarker and the mixture of faeces and the degradation of the compounds along the time could change the proxy values obtained with contemporary samples. These results were related with zooarchaeological studies where caprine remains were highly collected in that levels (Forgia et al., 2013). Additionally, the rock-shelter showed the rock-wall polished which could be related with the repeated rubbing of the ovicaprines housed in the pen to relieve the stinging caused by parasites (Vergès and Morales, 2016). All this

evidence strengthens the hypothesis obtained with the organic biomarkers. Nevertheless, a deeper study should be done to correlate the polished wall areas with those levels with the presence of ovicaprids according to the biomarkers.

Consequently, it is clear, that the concentration of phytosterols and Deoxy decrease from Middle Neolithic to the beginning of Early Middle Ages to increase again after the increment of pigs housed in the Vallone Inferno. This decrease was related to the decrease of the ovicaprid heads, and the increase of pigs housed in the rock-shelter. The same behaviour was observed in zooarchaeological studies (Forgia et al., 2021; Martín et al., 2023).

Principal component analysis was performed to detect possible grouping and similarities among the sediments. As presented in Fig. 3 (SiVi prefix has been eliminate from the samples nomenclature for a better illustration of the figure), the 91% of the samples were described with PC1 vs PC2 and three different clusters can be distinguished. One green, which corresponds to those samples with highest pig herding activity which correspond to those samples from to the Early Middle Ages; one yellow, which corresponds to those samples with lower pig herding activity and highest ruminant activity which correspond to Middle Neolithic and Bronze Age and finally, a purple group with those sample with very low concentration of the analytes which correspond to thermally altered samples. Loadings, influence, and explained variance are included in the supplementary material (Fig. 2S).

To sum up, livestock farming has been changing in the Vallone Inferno rock-shelter along the ages. At the Middle Neolithic ovicaprids were mainly enclosed in the rock-shelter and few pigs. This behaviour was changing gradually, and while the amount of ovicaprids decreased, the number of pigs increased until Early Middle Ages, where the pigs were the main species housed in the rock shelter. This behaviour agrees with zooarchaeological studies made in the Vallone Inferno (Martín et al., 2023).

According to all these results, similar studies have been done in El Mirador cave (Sierra de Atapuerca, Burgos, Spain) and in San Cristobal rock-shelter (Cantabrian mountain range, Araba, Spain) (Gea et al., 2017; Vallejo et al., 2022a, 2022b) The concentration of phytosterols were similar to those obtained in El Mirador cave at Neolithic and Bronze Ages, however, bile acids concentration were two times higher in Vallone Inferno (Vallejo et al., 2022a). Consequently, it is clear that the number of pigs was higher in Vallone Inferno than in El Mirador although in both cases was low at those ages, but it is not clear the number of ovicaprid heads. Considering bile acids, higher number of ovicaprids were enclosure in the Vallone Inferno but considering the phytosterols concentration, the number of ovicaprid were similar. Therefore, we need the zooarchaeological studies to determine really which of both approaches is the adequate. In the case of San Cristobal, all the concentrations are in the Vallone Inferno, therefore, higher number of ovicaprids were enclosure in the Vallone Inferno. Prost et al. (2017) made a similar study in four different German sites from different ages (Linearbandkeramik, Urn-field Period/Bronze Age, Iron Age, Roman Age). In all the cases, the concentrations were lower than in Vallone Inferno. These sites were not caves neither rock-shelter so the conservation of the biomarkers could be worse so lower concentration could be expected. Conversely, if the conservation the biomarkers was adequate, the number of pigs and ovicaprids was higher in Vallone Inferno than in those German sites.

3.3. Identification of the activity of shepherds

Recently, PG and Deox ratio was used to identify activities of the shepherds related with the livestock management such as the separation of the pregnant females or recent lamb ewes and their lambs from the herd or the use of the caves as herding caves (Vallejo et al., 2022a). Therefore, the concentration of several hormones and their metabolites were quantified to determine if Vallone Inferno rock-shelter was used for the same purposes.

Fig. 4 resumed the concentration of hormones and their metabolites obtained in the sample (also Table 1S for nominal values). As it is observed in Fig. 4, the hormones and metabolites concentration profile increased in SiVi20-SiVi18, SiVi15-SiVi13 and SiVi8 which were related with the sample with higher amounts of sterols/phytosterols and bile acids. Among these samples, SiVi20 and SiVi18 have the highest values.

According to PG and its metabolites (5βPT1 and 5βPTOH), their profiles were similar along the samples, however, their presence changed along the samples. PG was detected in all the samples, however, 5βPT1 and 5βPTOH just in those samples with high concentrations of faecal biomarkers (SiVi20-SiVi13 and SiVi8) and 5βPTOH also in SiVi21-SiVi24. The concentration of 5βPT1 was higher than PG when it was detected and, 5βPTOH concentration was lower. Therefore, both PG metabolites, 5βPT1 and 5βPTOH could be worked as biomarkers of hormonal activity. Besides, 5βPT1 and 5βPTOH could be used as alternative hormonal biomarkers to PG or as corroborative hormonal biomarker.

TT and its metabolite (Andro) were detected in SiVi20, SiVi19, SiVi17 and SiVi8 and, Andro also in SiVi15-SiVi13and SiVi26-SiVi28. These sample corresponds to those with high activity in the rock-shelter. Curiously, these compounds were not detected in SiVi18 where the concentration of the analytes related with the menstrual cycle and pregnancy was high. These two compounds are related with males, therefore, the absence of them could be related with the absence males in the enclosure. However, this hypothesis must be deeper studied.

Finally, E1 and E2 were detected for the first time. E1 was just detected in SiVi20 and SiVi18 (VII-IX century CE) which correspond to the samples with highest hormonal activity. Conversely, E2 was detected in the most modern samples with highest livestock activity SiVi20-SiVi17 (VII-IX century CE), SiVi15-SiVi13 (VII-VIII century CE) and in the prehistoric sample SiVi8 (half of the II millennium BCE - end of the Early Bronze – beginning of the Middle Bronze Age). These samples

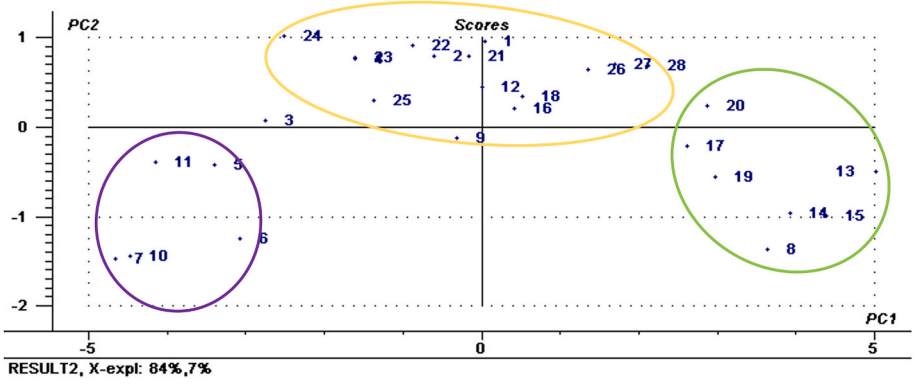

Fig. 3. Principal Component Analysis of the Vallone Inferno samples. SiVi prefix has been removed from the samples name to clarify the figure. Sterols, phytosterols and bile acid concentrations were normalised according to their logarithmic values to build the PCA. The missing values were filled with CLOD/2 values of each compound. These values were centred and normalised. Thermally altered samples are rounded in purple, lower pig herding activity and highest ruminant activity samples in yellow and highest pig herding activity samples in green.

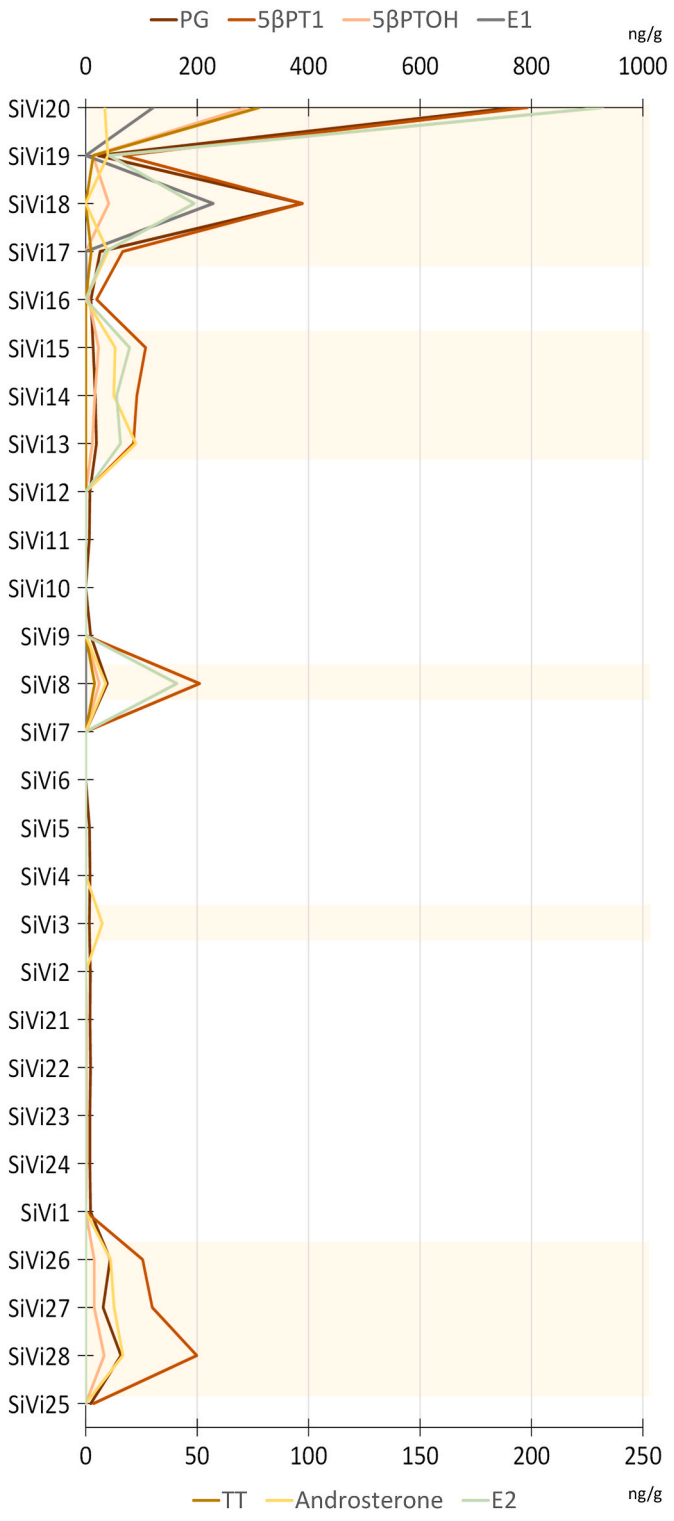

Fig. 4. Profile of nominal concentrations (ng/g) of hormones and hormone metabolite. Testosterone (TT), Androsterone and 17β-oestradiol (E2) are illustrated in a secondary axis (below). In light brown, the samples with highest herding activity are highlighted.

corresponded to those with highest pig activity in the rock-shelter, so it looks that the presence of these hormones was related with pig presence.

While for the early medieval period (VII-IX century CE), the relevance of pig presence in Sicily has been largely documented (Hartung, 2013), for the final stage of the Early and the beginning of the Middle Bronze Age, the predominance in the domestic assemblage, with pigs being the most abundant species, is a novelty in respect to very few known Sicilian Middle Bronze Age faunal assemblages (Platania, 2021; Martín et al., 2023). Nevertheless, our data can be linked to the change in the behaviour of mobility and trade of livestock in the Mediterranean and Aegean areas which has been detected with the first Italian pig haplotype outside of Italy at the beginning of the palatial period (14th century BCE) (Meiri et al., 2019). During this time, pigs from Near East are present in Greece, Sardinia and Sicily, while pigs from Europe were translocated east to Anatolia and the southern Levant, moreover an Italian pig haplotype has been discovered at Tiryns, Greece during the palatial period. At Vallone Inferno, and in similar still unknown places, a specialization in the breeding of pigs can be the response to the new demand conditioned by the Aegean trade behaviours.

Nevertheless, the increase of hormones concentration can be related with the increase of livestock heads. Thus, this effect must be eliminated normalizing the concentration of the hormones and their metabolites with the concentration of biomarkers related with the number of heads in the livestock. In the case of El Mirador cave, the concentration of PG was normalised with Deox because it is related with the number of the heads of ruminants (Vallejo et al., 2022a). However, in Vallone Inferno, the presence of pigs was significant so the normalisation just with Deox was not useful. The source of hormones can be both, ruminants, and pigs so the effect of both species must be corrected. Consequently, the normalisation of the hormone concentration was made with the sum of all the bile acids (Fig. 5 and Table 4S). The samples without faecal activity and with thermal alterations were eliminated from the profile.

All the ratios, increased considerably in SiVi20 and SiVi18 except TT and Andro in SiVi18. The increment of hormones ratios such as progesterone and their metabolites which are involved in the menstrual cycle and pregnancy of the species showed that in these samples the hormonal activity was considerably higher than in the rest. Therefore, during the VII-IX century CE, the rock-shelter could be used as breeding and birthing cave of pigs. Besides, in these two samples, the amount of TT/ΣBA and Andro/ΣBA decreased which are related with male activity. This could be related with the decrease of males in the sty or the increment of females comparing to males. Hyo concentration also decreased compared to SiVi19 and SiVi17 which could be related with the decrease of number of pigs or with the pig enclosure, females. Furthermore, zooarchaeological studies showed high number of deciduous teeth from immature pig individuals which in same chronologies which strengthens the hypothesis of the used of the rock-shelter as breeding and birthing cave for pigs at Early Middle Age (Forgia et al., 2013, 2021).

SiVi16, SiVi23-SiVi26 also showed a little increment of hormonal activity for PG/ΣBA and 5βPTOH/ΣBA. However, this increment was not enough to ensure a different herding management based on hormonal state of the herd. However, these results must be related with zooarchaeological studies.

These result can be compared with those obtained in El Mirador cave (Vallejo et al., 2022a). Ratio values obtained from El Mirador were higher than in Vallone Inferno which could be related with a higher hormonal activity. Nevertheless, main animal species are different, ovicaprids in El Mirador and pigs in the Vallone Inferno and the excretion of PG/Deox can be also different and a minimum change could be related with a high hormonal activity. Consequently, different studies should be done with contemporary samples of each animal species at different hormonal stages and scenarios to obtain a threshold value for each specie to ensure a high hormonal activity in the site.

4. Conclusions

Different sediment samples corresponding to Middle Neolithic and Early Middle Age, were collected from Vallone Inferno rock-shelter and analysed using a new method based on LC-MS analysis. The effectiveness of this methodology has been probed for the multianalysis of faecal biomarkers (β-sterol/phytosterol and bile acids), hormones and

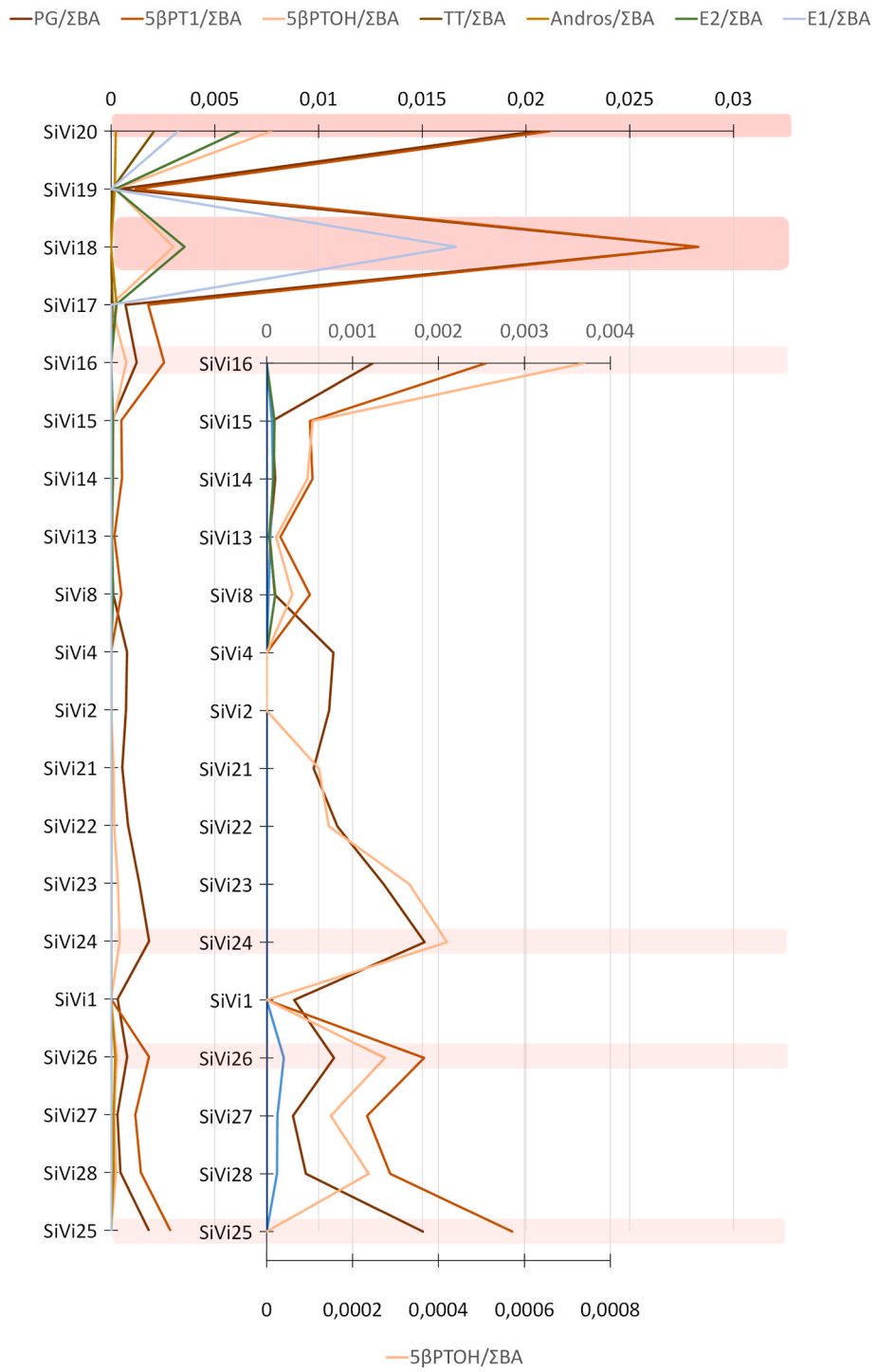

Fig. 5. Normalised hormones and metabolites profile. From sample SiVi16 to SiVi25, the figure has been enlarged and the axes have been changed for a better visualization. In red, high hormonal activity has been highlighted and in light red moderate/low hormonal activity.

hormone metabolites.

The quantification of those biomarkers and the use of different proxies have been useful to determine the livestock husbandry activity changes in the rock-shelter from Middle Neolithic to Early Middle Age. The Vallone Inferno rock-shelter was used as ovicaprid pen at Middle Neolithic with a low quantity of pigs. This distribution has been changed along the time to a rock-shelter was used as pig sty with a low quantity of ovicaprids. This reflects an incipient change in the management and use of the mountainous environment for the sustainment of pig farming. From our knowledge, this is the first time that this has been done using faecal biomarkers.

Additionally, we have demonstrated that PG and their metabolites biomarkers were useful again to identify human management of the livestock such as breeding and birthing rock-shelter for pigs. In this case, ratios between PG and the metabolites with ΣBA have been useful to identify high hormonal activity in Early Middle Age. PG is the most appropriate hormone for this purpose because it is more persistent than hormone metabolites and is identified together with faecal biomarkers.

Author contributions

Asier Vallejo: Conceptualization, Investigation, Validation, Writing - Original Draft, Review & Editing. Vincenza Forgia: Funding acquisition, Project administration, Visualization, Writing - Review & Editing. Josep Maria Vergès: Funding acquisition, Project administration, Review & Editing. Ane Gorostizu-Orkaiztegi: Formal analysis, Writing - Original Draft. Amaia Alday-Izaguirre: Data Curation, Writing - Review & Editing. Ainhoa Elejaga-Jimeno: Formal analysis, Writing - Review & Editing. M. Carmen Sampedro: Resources. Alicia Sánchez-Ortega: Resources, Supervision. Ramón J. Barrio: Funding acquisition, Supervision.

Data availability

The data set related to this manuscript can be found at https://doi.org/10.17632/pgwgf22tw8.1. This doi will not be active until the publication of the manuscript. If the reviewers want to check it, they can use this link: https://data.mendeley.com/datasets/pgwgf22tw8/draft?a=d7c9ea6d-74f7-4985-b924-3a63c20a6547.

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.quaint.2023.08.003.

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