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Two Hormones: Ghrelin and Leptin, Based on AMPK Signaling Pathway, Play a Role in Body Mass Control of *Eothenomys miletus* during Fasting in Kunming and Dali Regions

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ABSTRACT

Background: The ability to respond to global change and coexist with other species depends on phenotypic plasticity and physiological adaptation techniques of the same species living in various places differ according to the region.

Methods: *Eothenomys miletus* from Kunming (KM) and Dali (DL) under fasting and refeeding acclimation, we examined the thermogenic properties and the associated physiological indicators in the AMP dependent protein kinase (AMPK) pathway.

Result: The results demonstrated that energy consumption in *E. miletus* was decreased by fasting and that the process of survival adaption was significantly influenced by body mass, ghrelin concentration and AMPK activity. Following refeeding, pertinent physiological markers leveled off in the control group, demonstrating the high phenotypic plasticity of *E. miletus* and the critical role that leptin, ghrelin and AMPK pathways play in energy metabolism and environmental adaption during food fasting. Moreover, there may be a connection between geographical variations in physiological indicators under fasting conditions and variations in the ambient temperature and the food available to *E. miletus* in various places.

Key words: AMPK pathway, *Eothenomys miletus*, Fasting, Ghrelin, Leptin, Refeeding.

INTRODUCTION

Every wild animal is subject to the same conditions, which include cold and a lack of food, in this situation, energy metabolism and its control emerge as crucial elements of survival. Hormones and neurons interact to regulate energy metabolism, which in turn influences body mass, caloric intake and cell metabolism (Korhonen *et al.,* 2005). Taylor *et al*. (2013) found that it binds to the GHS-R1a growth hormone secretory receptor. White adipose tissue secretes leptin, which, by attaching to hypothalamic receptors, controls appetite and energy metabolism (Zhang *et al.,* 1994).

Studies have demonstrated that under fasting conditions, body mass, metabolic rate, ghrelin, leptin, hypothalamic appetite related neuropeptides and AMP activated protein kinase (AMPK) signal pathway related markers would change correspondingly. The hypothalamic AMPK signal pathway is one of them and it is essential for controlling animal feeding (Andersson *et al.,* 2004). In the hypothalamus, inhibiting AMPK activity would result in anorexia and body mass loss, according to *Rattus norvegicus* research findings, fasting raised AMPK activity in several hypothalamic areas, stimulated feeding and increased body mass (Briski *et al.,* 2020).

In order to regulating the release of neuropeptides, ghrelin and leptin can work together to encourage and inhibit food intake in the hypothalamus (Zhang *et al.,* 1997). To regulate food intake and energy metabolism, they can also manipulate the AMPK signal pathway (Stark *et al.,* 2013). Through raising AMPK activity, ghrelin suppresses the expression of malonyl-CoA and the phosphorylation of acetyl-CoA carboxylase (ACC). CPT-1 is subsequently inhibited, leading to food intake (Kohno *et al.,* 2008). In addition, cocaine and amphetamine regulated transcription peptide (CART) and proopiomelanocortin (POMC), Agouti related protein (AgRP) and neuropeptide Y (NPY) all participate in the regulation of AMPK (Banerjee *et al.,* 2018).

Hengduan Mountains and its surrounding areas are home to a large population of *Eothenomys milletus*, an endemic species to China (Zhu *et al.,* 2010). Results from our earlier studies demonstrated that *E. miletus* under random food restriction exhibited phenotypic plasticity in their

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physiological index and the physiological indicators revealed regional heterogeneity (Ren *et al.,* 2020). It is currently unknown how ghrelin, leptin and AMPK signaling pathways affect the control of body mass. Will these indicators then reveal regional variations following brief fasting and refeeding in various regions? We showed the phenotypic changes of ghrelin, leptin, hypothalamic appetite-related neuropeptides, AMPK signaling pathway and other indicators of *E. miletus* in two regions in order to illustrate the survival adaptation strategies of *E. miletus* under fasting conditions. This provides a theoretical basis for the investigation of tiny animals' methods of environmental adaption in the Hengduan Mountains.

MATERIALS AND METHODS

Animals and experimental design

In KM (102.80°E, 24.88°N, height 2020 m, n=18) and DL (99.90°E, 26.53°N, altitude 2590 m, n=18), a total of 36 *E. miletus* were captured in mouse traps. *E. miletus* were transported back to the Yunnan Normal University lab where they were kept alone in a clear plastic box (260 mm × 160 mm × 150 mm) at a temperature of 25±1°C with an 8L:16D photoperiod. Detailed processing methods was in Liu *et al*. (2022). All animal procedures were within the rules of Animals Care and Use Committee of School of Life Sciences, Yunnan Normal University. This study was approved by the committee (13-0901-011).

Six groups of animals were formed by randomly assigning them to the control group (C-KM, C-DL), the 10-hour fasting group (F10h-KM, F10h-DL) and the 10-day refeeding group (Re10d-KM, Re10d-DL). On the first day of the experiment, after 10 hours of fasting and after 10 days of refeeding, the animals were killed by giving them pentobarbital sodium (50 mg/kg) after measure RMR, food intake and body mass. When indicators could be evaluated later, organs were taken out, blood were collected, the hypothalamus were stripped and then maintained them in a freezer (-80°C).

Measurement of body mass, body composition and food intake

Food consumption was measured by food balance method and specific methods of determining body composition could be found in Zhu *et al*. (2010). Body mass was measured utilizing an LT502 electronic weighing device (accurate to 0.01 g).

Measurement of RMR

The RMR was calculated using an 8-channel FMS portable respiratory metabolic monitoring system (Sable Systems International, Inc., USA) after fasted for three to four hours. Details on the determination method are provided in Li *et al*. (2005).

Determination of leptin, total ghrelin content, AMPK activity, malonyl-COA activity, CPT-1 activity and neuropeptide expression in medial hypothalamus

Measurements was details in Liu *et al*. (2022). AMPK activity, malonyl-COA activity, CPT-1 activity, serum leptin and total ghrelin content and stomach ghrelin content were measured by enzyme-linked immunosorbent assay (ELISA) kits. Total RNA was extracted from the medial hypothalamus using the TRIzol Kit. Measurements was details in Zhu *et al*. (2015).

Data analysis

Software called SPSS 22.0 was used to analyze the data. Data were checked for homogeneity and norality of variance using the Kolmogorov-Smirnov and Levene tests, respectively, before any statistical analyses. All of the data were combined and counted because there were no physiological markers that distinguished the different sexes of *E. miletus* in either region appreciably. A difference of significance of $P<0.05$ is shown and the results are presented as means± SE. Measurements was details in Liu *et al*. (2022).

RESULTS AND DISSICUSION

Body mass, RMR and body composition

The body mass was significantly influenced by region and fasting (Region: $F_{1,30}=5.208$, P=0.03; Fasting: $F_{2,30}=4.828$, P=0.015; Region×Fasting: $F_{2,30}=0.348$, P=0.709). RMR was significantly influenced by both region and fasting (Region: $F_{1,30}=23.866$, P=0.000; Fasting: $F_{2,30}=13.972$, P=0.000; Region×Fasting: $F_{2,30}=0.012$, P=0.988). RMR in both of the two locations reached the control group's level after refeeding (Fig. 1a, b). Large intestine wet mass without content: $F_{2,30}=10.078$, P=0.000; Large intestine dry mass: $F_{2,30}=10.158$, P=0.000; Cecum wet mass without content: $F_{2,30}=5.744$, P=0.008; Cecum dry mass: $F_{2,30}=6.834$, P=0.004; all of which were significantly impacted by the correlation between region and fasting. Additional data on the digestive system and body composition showed no appreciable alterations (Table 1).

Animals will alter their body composition or employ various coping mechanisms in response to the scarcity of food sources. According to research, hunger will have an impact on an animal's ability to produce heat and change their body mass (Ren *et al.,* 2020). The results demonstrated that body mass and RMR in *E. miletus* reduced after fasting but restored to the control level after feeding. Internal organs can exhibit phenotypic alterations that may be a result of their functional adaptation (Wang *et al.,* 2003). Such as AMPK activity in liver is related to energy storage (Liddle, 2019). Animals' intestinal and cecum decant masses dramatically decreased after fasting, reflecting adaptive alterations in *E. miletus's* digestive capacity to handle variations in nutrition, energy intake, or other aspect under extreme circumstances (Bonin *et al.,* 2016). Our findings show that *E. miletus* in the DL region lost greater amounts of body mass after fasting than KM, suggesting that the area's winter food supply was limited and that they were able to adjust their body composition as needed to react to the changes in the food environment.

Changes of serum leptin, serum ghrelin, stomach ghrelin expression and AMPK, malonyl-COA and CPT-1 activities in hypothalamus

Serum leptin, stomach ghrelin and serum ghrelin content varied significantly by region (Leptin: $F_{1,30}=57.696$, P=0.000;

Table 1: Changes in body composition and the digestive system in various locations while fasting of *E. miletus.*

Parameters	KM			DL			Statistical summary					
							Region		Fasti		Region × fasting	
	C_1-KM (n=6)	F_{10h}-KM (n=6)	Re_{10d}-KM (n=6)	C_1-DL (n=6)	F_{10h}-DL (n=6)	Re_{10d}-DL (n=6)	$F_{1,30}$	P	$F_{2,30}$	P	$F_{2,30}$	P
Carcass (g)	32.77±1.92	32.67±1.69	34.43±1.92	29.01±0.81	26.87±2.18	32.83±1.52	2.157	0.153	1.576	0.224	0.382	0.686
Heart wet mass (g)	0.28±0.02	0.22±0.01	0.28±0.02	0.23±0.02	0.20±0.01	0.28±0.02	2.406	0.132	4.991	<0.05	0.688	0.511
Liver wet mass (g)	3.84±0.37	2.03±0.12	3.21±0.27	3.09±0.18	1.67±0.08	2.61±0.33	4.169	0.050	14.249	<0.001	0.486	0.620
Spleen wet mass (g)	0.13±0.02	0.09±0.02	0.13±0.02	0.12±0.02	0.09±0.01	0.10±0.008	1.640	0.210	2.009	0.152	0.221	0.803
Lungs wet mass (g)	0.29±0.03	0.29±0.03	0.36±0.03	0.22±0.01	0.29±0.03	0.31±0.01	0.914	0.347	5.892	<0.05	1.478	0.24
Kidney wet mass (g)	0.42±0.02	0.43±0.02	0.45±0.02	0.38±0.02	0.38±0.02	0.42±0.03	1.987	0.169	1.671	0.206	0.050	0.951
Stomach wet mass with content (g)	0.68±0.06	0.41±0.11	0.71±0.05	0.74±0.09	0.26±0.01	0.57±0.09	1.815	0.188	11.381	<0.001	1.224	0.309
Large intestine wet mass with content (g)	0.72±0.11	0.58±0.04	0.70±0.12	0.71±0.09	0.49±0.06	0.89±0.12	0.093	0.762	2.906	0.071	1.071	0.356
Small intestine wet mass with content (g)	2.11±0.05	1.62±0.12	1.99±0.13	1.69±0.11	1.55±0.09	1.82±0.06	2.684	0.112	2.276	0.121	2.730	0.082
Cecum wet mass with content (g)	1.84±0.09	2.18±0.12	1.88±0.23	1.53±0.35	1.92±0.20	1.95±0.31	1.075	0.308	0.787	0.465	0.441	0.648
Stomach wet mass with no conten t(g)	0.18±0.02	0.25±0.03	0.19±0.02	0.22±0.05	0.21±0.01	0.25±0.008	0.318	0.577	0.505	0.609	1.634	0.21
Large intestine wet mass with no content (g)	0.36±0.02	0.47±0.04	0.44±0.03	0.45±0.03	0.30±0.02	0.43±0.04	1.991	0.169	1.706	0.199	10.078	<0.001
Small intestine wet mass with no content (g)	0.94±0.13	0.90±0.06	1.05±0.08	0.91±0.05	0.76±0.06	1.03±0.08	3.129	0.087	5.735	<0.05	0.720	0.495
Cecum wet mass with no content (g)	0.48±0.05	0.49±0.02	0.46±0.03	0.63±0.03	0.43±0.03	0.49±0.02	3.769	0.062	4.182	<0.05	5.744	<0.05
Large intestine length (cm)	19.40±1.54	21.38±0.67	22.48±0.91	22.83±0.74	19.68±1.52	22.63±0.47	0.351	0.558	1.722	0.197	2.929	0.069
Small intestine length (cm)	36.42±2.39	37.32±1.2	39.07±1.76	35.17±0.98	36.45±1.19	36.55±0.89	0.341	0.564	1.254	0.300	0.331	0.721
Cecum length (cm)	9.33±0.71	10.88±0.90	12.05±0.60	9.48±1.04	11.5±1.13	11.57±0.48	0.088	0.768	4.373	<0.05	0.250	0.780
Heart dry mass (g)	0.06±0.005	0.06±0.003	0.07±0.005	0.08±0.018	0.06±0.004	0.07±0.004	0.384	0.540	0.582	0.565	0.658	0.525
Liver dry mass (g)	1.26±0.12	0.70±0.04	1.11±0.11	1.01±0.07	0.54±0.02	0.96±0.09	4.323	<0.05	13.346	<0.001	0.240	0.788
Spleen dry mass (g)	0.03±0.004	0.02±0.004	0.03±0.005	0.03±0.006	0.03±0.004	0.03±0.001	0.359	0.554	1.376	0.269	0.507	0.608
Lungs dry mass(g)	0.09±0.01	0.08±0.01	0.11±0.02	0.06±0.002	0.08±0.008	0.09±0.005	1.115	0.300	3.531	0.042	2.305	0.118
Kidney dry mass(g)	0.14±0.008	0.14±0.006	0.13±0.007	0.12±0.006	0.13±0.007	0.15±0.01	0.000	0.998	1.106	0.344	2.761	0.080
Large intestine dry mass with content (g)	0.07±0.004	0.10±0.01	0.09±0.005	0.10±0.007	0.07±0.005	0.09±0.004	0.699	0.410	0.188	0.830	10.158	<0.001
Small intestine dry mass with content (g)	0.21±0.03	0.18±0.02	0.18±0.01	0.16±0.01	0.15±0.02	0.21±0.01	3.689	0.065	2.927	0.070	2.389	0.110
Cecum dry mass with content (g)	0.10±0.01	0.11±0.01	0.09±0.004	0.11±0.005	0.08±0.007	0.10±0.006	1.487	0.233	1.557	0.228	6.834	<0.05

Data are means±SE.

Stomach ghrelin: $F_{1,30}$=104.747, P=0.000; Serum ghrelin: $F_{1,30}$=38.022, P=0.000). The effects of fasting on serum leptin, stomach ghrelin and serum ghrelin levels were all profound in *E. miletus* (Leptin: $F_{2,30}$=23.993, P=0.000; Stomach ghrelin: $F_{2,30}$=35.790, P=0.000; Serum ghrelin: $F_{2,30}$=25.137, P=0.000). The hypothalamic CPT-1 activity in *E. miletus* was only substantially influenced by the region ($F_{1,30}$=4.190, P=0.050). Fasting had a significant impact on the hypothalamic activity of AMPK, malonyl-COA and CPT-1 (AMPK: $F_{2,30}$=29.936, P=0.000; Malonyl-CoA: $F_{2,30}$=14.463, P=0.000; CPT-1: $F_{2,30}$=37.872, P=0.000). After the *E. miletus* in both locations had been removed on the tenth day, the serum leptin levels and the activities of AMPK, malonyl-COA and CPT-1 reverted to those of the control group (Fig 1 c, d, e, f, g, h).

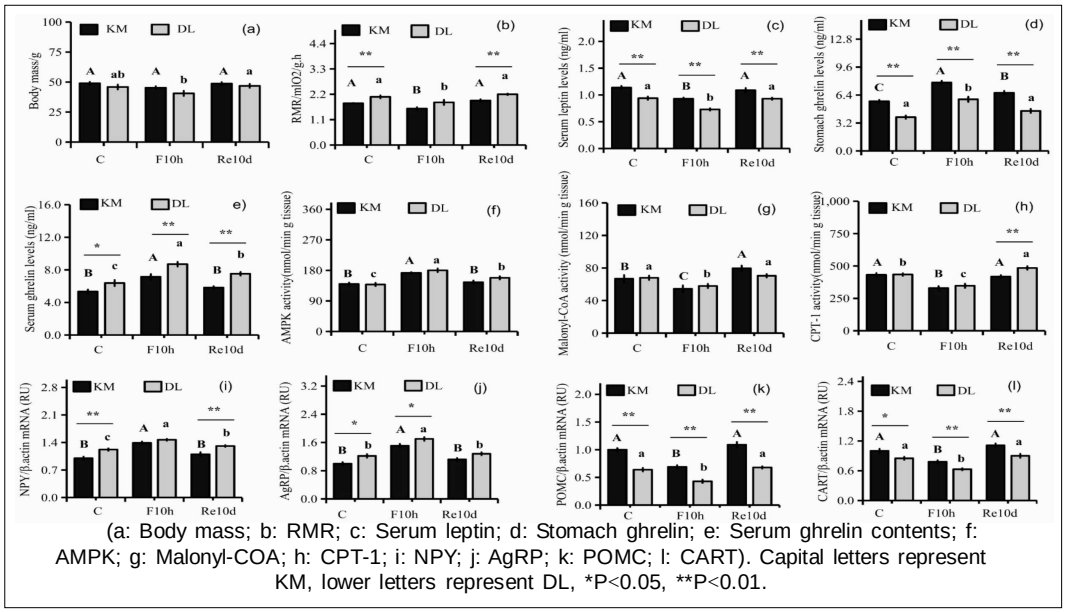
Dietary intake will limit ghrelin secretion and dietary restriction will boost it (Bake *et al.,* 2020). In the present investigation, following fasting, serum leptin concentration fell whereas stomach ghrelin and serum ghrelin concentration increased. It is worth noticing that the content of ghrelin in serum of *E. miletus* after fasting was higher than that in stomach. Our explanation for this phenomenon is that fasting stimulated *E. miletus* greatly and fasting time was short. After ghrelin was produced in stomach fundus, it acted on hypothalamus rapidly through blood circulation. Therefore, a large number of ghrelin could be detected in serum at this time. After refeeding, the feeding center of hypothalamus received the regulation of satiety signal and then reduced ghrelin and increased the secretion of leptin. Leptin and ghrelin levels were also greater in KM *E. miletus* than in DL, which can be explained by the superior wintertime feeding conditions in KM. In order to control body mass and waster of energy in the event of a sudden food deficit, KM *E. miletus* would release more ghrelin and leptin (Ren *et al.,* 2020).

AMPK activity increased whereas malonyl-COA and CPT-1 activities decreased in the hypothalamus. This is because in the hypothalamus, the increasing of food intake attribute to the exogenous activation of AMPK, while exogenous inhibition reduces it. In the meantime, AMPK activity increased because of the food restriction in the hypothalamus while refeeding decreases it (Lopez *et al.,* 2008). Fasting can swiftly boost ghrelin production after activating AMPK, which can subsequently stop ACC from being phosphorylated, lowering the concentration of malonyl-CoA and reducing the release of CPT-1. The secretion concentration of serum leptin is proportional to the level of fat, while leptin suppresses the activity of hypothalamic AMPK, which lowers food intake (Stark *et al.,* 2013).

Changes of neuropeptide expressions in hypothalamus

Fasting significantly influenced the level of NPY, AgRP, POMC and CART expression in *E. miletus* (NPY: $F_{2,30}$=37.907, P=0.000; AgRP: $F_{2,30}$=82.157, P=0.000; POMC: $F_{2,30}$=38.658, P=0.000; CART: $F_{2,30}$=27.030, P=0.000); similarly, the influence of region on NPY, AgRP, POMC and CART expression in E. After another 10 days of feeding, the expression of AgRP and POMC in both regions returned to the same state as in the control group (Fig 1 i, j, k, l).

Leptin production is decreased during fasting, allowing ghrelin to trigger the activation of CPT-1a and CPT-1c, which in turn activates NPY/AgRP neurons. This process is made possible by the lowering of malonyl-COA caused by GHS-R1a-AMPK. However, hunger and body mass loss result



(a: Body mass; b: RMR; c: Serum leptin; d: Stomach ghrelin; e: Serum ghrelin contents; f: AMPK; g: Malonyl-COA; h: CPT-1; i: NPY; j: AgRP; k: POMC; l: CART). Capital letters represent KM, lower letters represent DL, *P<0.05, **P<0.01.

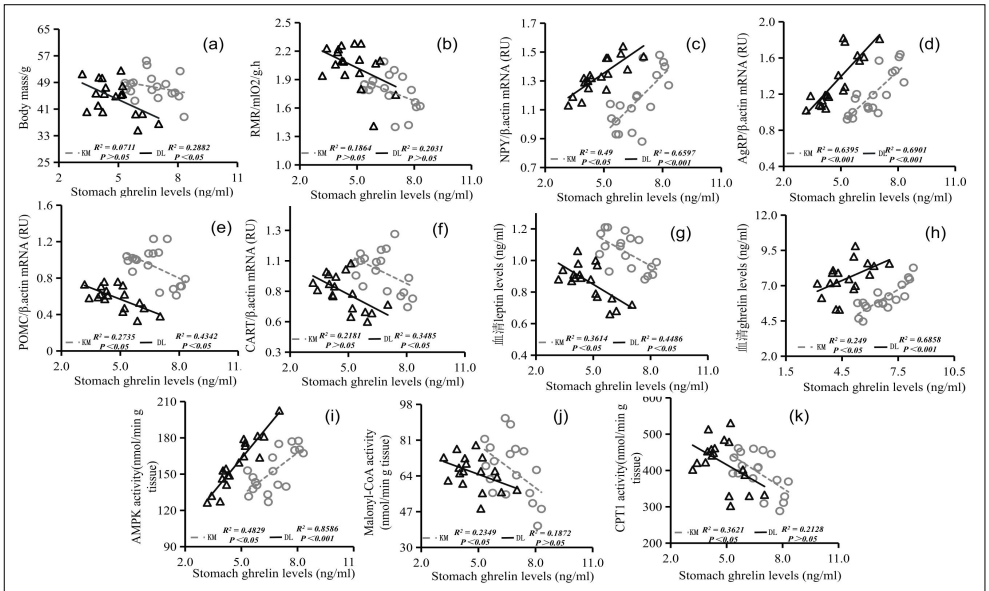
Fig 1: Changes of body mass, RMR, serum leptin contents, stomach ghrelin contents, serum ghrelin contents, AMPK activity, malonyl-COA activity, CPT-1 activity, NPY expression levels, AgRP expression levels, POMC expression levels and CART expression levels of *E. miletus* under fasting conditions.

after gene deletion of AgRP neurons (Gropp *et al.,* 2005; Luquet *et al.,* 2005). Neurons that produce AgRP will also be unable to adequately perform this task due to the impairment of AMPK activation (Claret *et al.,* 2007). While feeding-inhibiting neuropeptide synthesis was higher in KM than in DL, feeding-promoting neuropeptide synthesis was higher in DL. Our argument for this is that DL has poor feeding conditions in the winter due to its high altitude. In order to survive in a generally hostile environment, DL *E. miletus* swiftly released hunger signals to boost appetite and began foraging in the midst of food shortages.

Correlation analysis

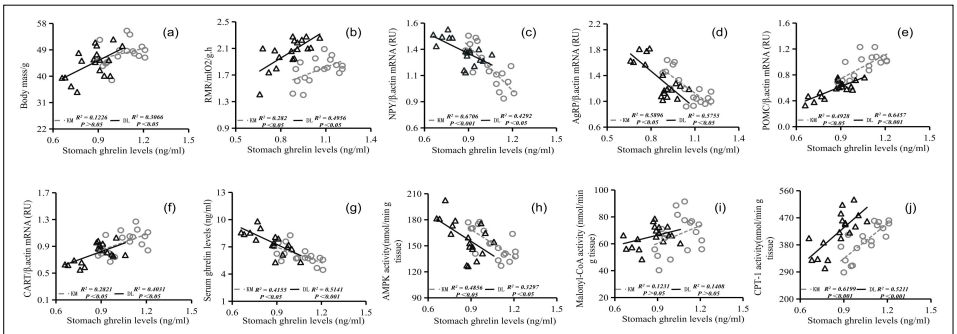
NPY expression, AgRP expression, serum ghrelin content and AMPK activity were all positively correlated with stomach ghrelin content, while body mass, RMR expression, POMC expression, CART expression, serum leptin content, malonyl-COA activity and CPT-1 activity were all negatively correlated (Fig 2). Malonyl-COA activity, CPT-1 activity, POMC expression, CART expression, serum leptin content and body mass were all positively correlated with serum leptin content, while NPY expression, AgRP expression, serum ghrelin content, AMPK activity and CPT-1 expression were all negatively correlated (Fig 3). NPY expression, AgRP expression and AMPK activity were all positively correlated with serum ghrelin content, while body mass, RMR, POMC expression, CART expression, malonyl-COA activity and CPT-1 activity were all negatively correlated (Fig 4).

It is also important to note that ghrelin and leptin did not function independently throughout the entire experiment. They act as opposing hormones to anorexia on the one hand



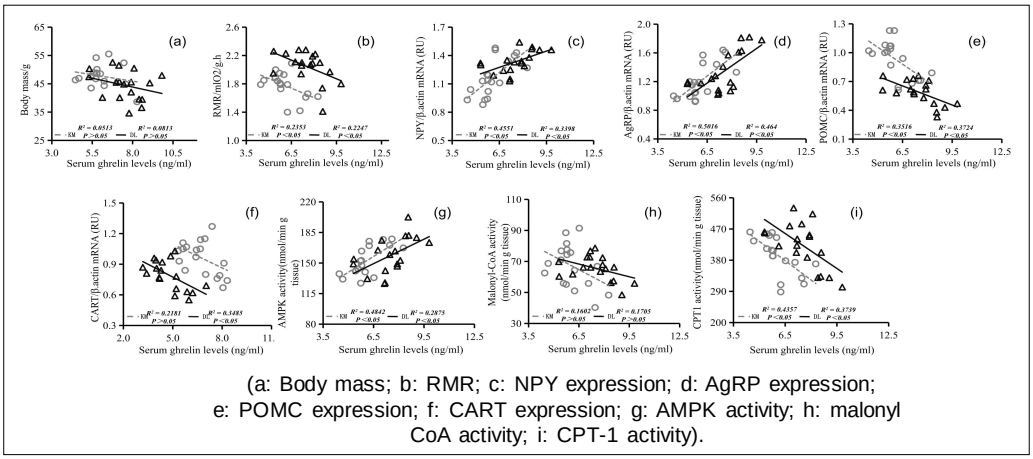
(a: Body mass; b: RMR; c: NPY expression; d: AgRP expression; e: POMC expression; f: CART expression; g: Serum leptin content; h: Serum ghrelin content; i: AMPK activity; j: malonyl-CoA activity; ; k: CPT-1 activity).

Fig 2: Correlation between stomach ghrelin content and other indicators in *E. miletus* under fasting conditions.



(a: Body mass; b: RMR; c: NPY expression; d: AgRP expression; e: POMC expression; f: CART expression; g: Serum ghrelin content; h: AMPK activity; i: Malonyl-CoA activity; j; CPT-1 activity).

Fig 3: Correlation between serum leptin content and other indicators in *E. miletus* under fasting conditions.



(a: Body mass; b: RMR; c: NPY expression; d: AgRP expression; e: POMC expression; f: CART expression; g: AMPK activity; h: malonyl CoA activity; i: CPT-1 activity).

Fig 4: Correlation between serum ghrelin content and other indicators in *E. miletus* under fasting conditions.

and anorexia on the other. In order to conserve energy and boost hunger when food is in short supply, leptin concentration decreases and ghrelin levels rise (Shintani *et al.,* 2001). Even if the ghrelin concentration stays constant, the fall in leptin concentration is sufficient to maintain the increased food intake in winter, leptin is the main blocker of hypothalamic ghrelin activity, according to Kalra's hypothesis (Kalra *et al.,* 2003). Contrarily, leptin was also found in stomach (Bado *et al.,* 1998), despite earlier theories that it originated from fatty tissue (Zhang *et al.,* 1994). These investigations demonstrate that the AMPK signaling pathway, in which ghrelin plays an important role, is maintained by the combined action of leptin and ghrelin, which is the key to maintaining appetite (Kohno *et al.,* 2008). We also discovered that ghrelin and leptin may cooperate in the current study to keep AMPK signal active in order to control energy metabolism in *E. miletus.*

CONCLUSION

In conclusion, fasting decreased body mass and the RMR, elevated ghrelin, lowered blood leptin, activated the hypothalamus-mediated AMPK signal pathway and decreased CPT-1 with elevated oxidation in *E. miletus*. Changes in ghrelin and leptin levels would cause the expression of NPY and AgRP to gradually increase along with dietary intake. In the hypothalamus, AMPK signaling pathway, ghrelin and leptin may act swiftly to play a role through their receptors and control feeding to ensure that *E. miletus* maintains homeostasis with energy when under food stress. Furthermore, the body mass regulation of *E. miletus* from DL was more sensitive, which may have been a result of the region's limited food availability and harsh winter weather.

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Conflict of interest: None.

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