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Original Article

The effect of water temperature on food transit time and digestive enzymes activity in Caspian kutum (*Rutilus kutum*) larvae

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Abstract: The present study investigates the effects of water temperature on digestive enzymes activity and food transit time in Caspian kutum (*Rutilus kutum*) larvae. Caspian kutum larvae (532 ± 0.05 and 543 ± 0.02 mg) were divided into two groups with three replicates and reared at different water temperature *i.e.* 25.6 ± 0.4°C (T_1) and 18.4 ± 0.1°C (T_2). At the end of the experiment, sampling of intestine was performed at 0, 1, 3, 5, 8, 16, 24 and 30 h after feeding from each treatment. In T_2, food was observed until 24 h after feeding and the intestine was empty 29 h after feeding, while in T_1 19 h after feeding the intestine was empty. Digestive enzymes activities were higher in T_2 treatment. The peaks of trypsin and alkaline phosphatase enzymes activity were found 8 h after feeding in T_1, while occurred 16 h after feeding in T_2. The highest chymotrypsin and alpha-amylase enzymes activity were observed 5 and 8h after feeding in T_1 and T_2, respectively. These results confirmed remarkable effects of temperature on food transit time and digestive enzymes activity of Caspian kutum.

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Introduction

The *Rutilus kutum* is an important commercial fish species in the Caspian Sea that faced a sharp decline of natural population due to overfishing, water pollution, and loss of habitat and spawning grounds (Hoseinifar et al., 2014). The severe decline in natural population motivated the Iranian government to launch its restocking project in 1984 by releasing the fingerlings obtained from artificial propagation to the rivers of southeastern region of Caspian Sea. At present, around 200 million of fry (~ 1 g) are annually released to southern rivers for stocks enhancement purposes (Caspian Environment. org, 2007).

The passage of food in fish gut is depend on a number of factors including species, fish age/size, water temperature, food quality, and feeding frequency (Temming and Herrmann, 2001; Specziár, 2002; Wuenschel and Werner, 2004). In particular, water temperature has a significant effect on food transit time that as it can affects digestive enzymes activity and feed intake (Temming and Herrmann, 2001; Kofuji et al., 2005). Nakada (2000) reported remarkable increase of food transit time in Japanese yellowtail (*Seriola quinqueradiata*) following decrease of water temperatures. Also decreased digestion rate in the same species during the cold months has been reported (Kofuji et al., 2005) which results in lower apparent protein digestibility (Kofuji et al., 2005).

Type and function of the digestive enzymes clearly affect digestive process and is a very important issue in the study of the digestive physiology (Gisbert et al., 2009). Moreover, efficiency of the enzyme function and digestive process is related to important factors such as sufficient time for hydrolysis of food and sufficient amount of enzymes in the gut (Olsson and Holmgren, 2001; Uscanga et al., 2010). The first parameter is determined by measurement of the food transit time.

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Due to seasonal variation, the Caspian kutum (*R. kutum*) is exposed to different water temperatures throughout the year, which may affects food transit time, food intake and digestive enzyme activity. Therefore, the purpose of this study was investigation of the effects of water temperature on food passage time and digestive enzymes activity in Caspian kutum.

Materials and methods

Experimental design and management: The present study was performed in August and December 2012 at Shahid Fazli Aquaculture Research Station of Gorgon University (Golestan Province, Iran). In August and December, average water temperature of the tanks were 25.6 ± 0.4°C and 18.4 ± 0.1°C, respectively. The Caspian kutum larvae were supplied from the Sijaval fish farm (Golestan Province, Iran) and acclimated to laboratory conditions for 2 weeks. During acclimation, fish were fed twice a day with a commercial formulated diet (Biomar, 0.8 mm). After acclimation, a total of 360 Caspian kutum including 180 specimens (532 ± 0.05 mg) in 25.6± 0.4°C (T_1) and 180 specimens (543 ± 0.02 mg) in 18.4±0.1°C (T_2) were used, respectively in three replicates fiberglass tanks (420 L capacity with 300 L water volume with 60 fish per tank). There were no significant differences between weights of fishes in both treatments (P>0.05). The photoperiod condition was similar in both treatments. Also water quality parameters such as pH, dissolved oxygen, ammonia, nitrate and nitrite had no significant difference in two treatments (P>0.05).

Determination of gut transit time: Food transit time was determined according to Miegel et al. (2010) by visual observations of the digestive tract contents at 0, 1, 3, 5, 8, 16, 24 and 30 h post feeding. This was performed by monitoring the passage of feed and digesta through the entire digestive tract in dissected intestines.

Sampling and preparation of crude enzyme extract: Digesta, whole gut and intestinal tissue samples were collected by dissection at different times post feeding as mentioned above. Half-hour after feeding, the rearing tanks were cleaned to remove remaining food. Three fish were randomly selected from each tank. The entire digestive tract (from esophagus to the anus) was removed and adipose tissues were cautiously cleaned. Afterward samples were rinsed with chilled refined water and stored at −80°C for further analysis (Harpaz et al., 2005; Perez-Jimenez et al., 2009). Prior to assessment, the stored samples were partially thawed, weighed and homogenized in five volumes of 0.2 M NaCl solution (w/v) using an electric homogenizer (IKA T25 digital, Ultra Turrax model). The tip of the homogenizer was cleaned for each dissimilar homogenizing process with distilled water and wiped with tissue paper (Gawlicka et al., 2000). The suspensions were centrifuged for 30 min at 10,000 g, 4°C using a refrigerated centrifuge (Eppendorf 5810R) (Klomklao et al., 2007). Thereafter, the supernatants were softly pipetted into separate sterile centrifuge vials and frozen at −80°C until enzyme activity or protein content analysis. Sample preparations were conducted at low temperature by working on ice.

Enzyme assays: The trypsin (EC 3.4.21.4) activity was estimated using benzoyl-DLarginine-p-nitroanilide (BAPNA) as substrate according to Erlanger et al.(1961) and absorbance was read at 410 nm using spectrophotometer (lightwave-S2000 UV/VIS). Chymotrypsin (EC 3.4.21.1) activity was determined after Hummel (1959) with BTEE (N-benzoyl-L-tyrosine ethyl ester) as substrate and absorbance was read at 256 nm using spectrophotometer (lightwave-S2000 UV/VIS). The activity of α-amylase (EC 3.2.1.1) was calculated using starch as the substrate according to Bernfeld (1951) and absorbance was read at 540 nm using spectrophotometer (lightwave-S2000 UV/VIS).

Alkaline phosphatase (EC 3.1.3.1) activity was assayed as previously described (Bessey et al., 1946) at 37°C using 4-nitrophenyl phosphate (PNPP) as

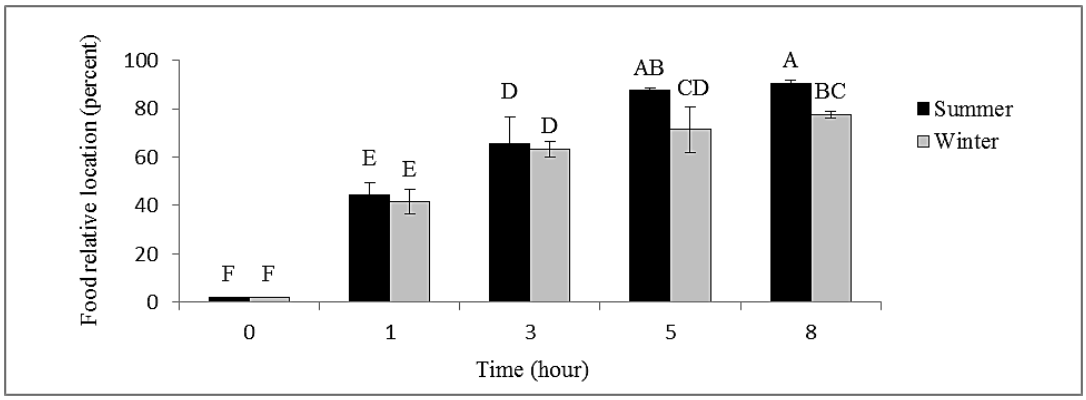

Figure 1. The relative location of food after feeding in the gut (T_1: Summer and T_2: Winter). Bars assigned with the same letter are significantly differed ($P< 0.05$); Values are presented as the mean ± S.E.

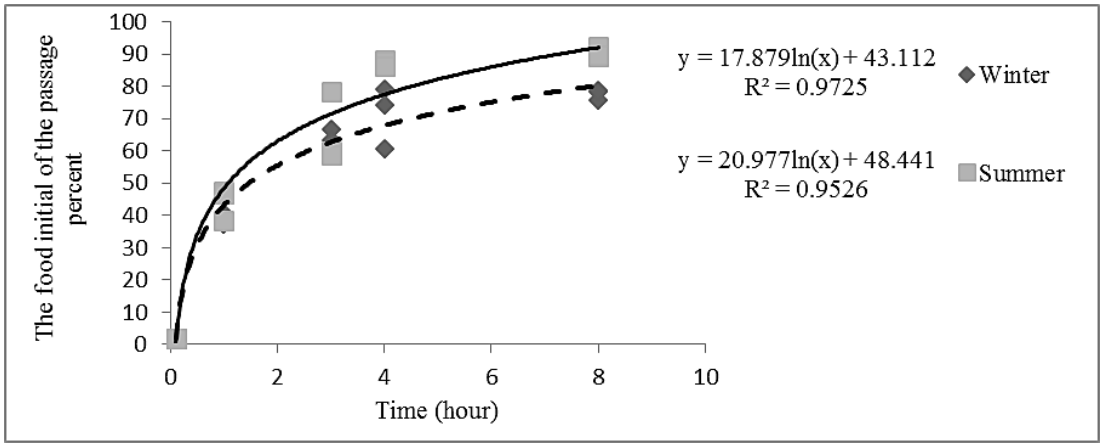

Figure 2. The passage percent of initial section of food in the intestine of Caspian kutum reared in different water temperature (T_1: Summer and T_2: Winter).

substrate and absorbance was read at 407 nm using spectrophotometer (lightwave-S2000 UV/VIS).

Bovine serum albumin solution (BSA) was used as the standard in the determination of soluble protein (Bradford, 1976). All enzymes activities were expressed as specific activity (U mg protein^{-1}). All enzymatic assays were performed at 25 °C (except alkaline phosphatase at 37°C) in triplicate.

Statistical analysis: The study of food transit was performed by split-plot completely randomized design in time (5 levels: 0, 1, 3, 5, 8 h) with two treatments. Also for the food location changes in the gut, the regression analysis was used. The study of digestive enzymes activity and protein was performed by split-plot completely randomized design in time (7 levels: 0, 1, 3, 5, 8, 16 and 24 h) and (8 levels: 0, 1, 3, 5, 8, 16, 24 and 30 h) in T_1 and T_2, respectively. When F values were significant ($P<0.05$), means in a treatment in different times were compared with LSD test and in two treatment in a time were compared with T-test. Statistical analyses were done using SPSS software. Results are expressed as mean ±SE.

Results

Food transit time: There results of water temperature effects on food transit time are presented in Figures 1-3. The results showed that the changes in water temperature significantly affected food transit time ($P<0.05$). The influence of the treatment, time and their interaction on food passage in the gut was significant ($P<0.05$).

The results of the LSD test showed there was no significant difference between the food relative location in two treatments up to 3 h post feeding ($P>0.05$). However, at 5 and 8 h post feeding sampling significant difference was noticed ($P<0.05$) (Fig. 1). According to the results of the

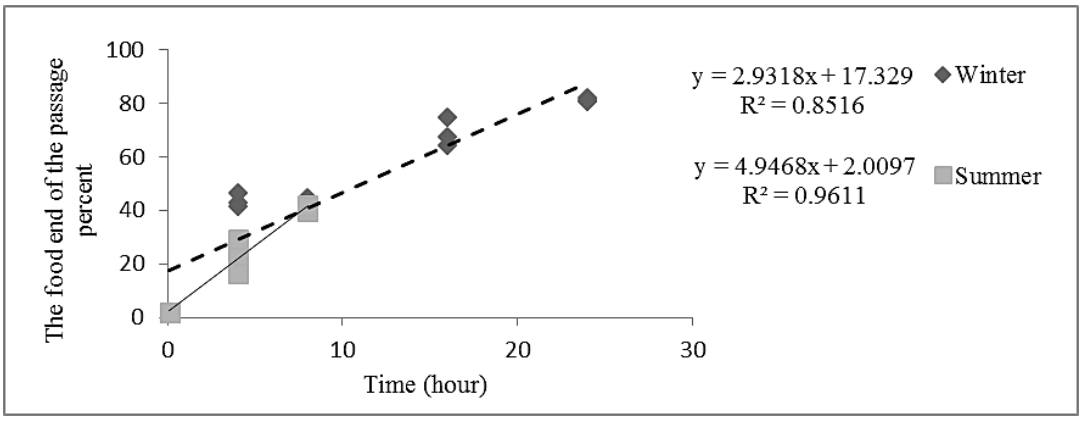

Figure 3. The passage percent of last section of food in the intestine of Caspian kutum reared in different water temperature (T_1: Summer and T_2: Winter).

Table 1. Changes in soluble protein concentration (mean ± SE, n=3) post feeding in Caspian kutum reared in different water temperature (T_1: Summer and T_2: Winter).

Time/protein	Treatment 1	Treatment 2
0	1.74 ± 0.09^{d} A	1.65 ± 0.14^{c} A
1	3.07 ± 0.31^{c} A	1.48 ± 0.11^{c}B
3	4.77 ± 0.6^{a} A	2.49 ± 0.25^{ab} B
5	3.17 ± 0.4^{ab} A	2.04 ± 0.5^{bc} A
8	4.26 ± 0.31^{ab} A	1.89 ± 0.4^{c} B
16	4.36 ± 0.08^{a} A	2.42 ± 0.01^{b} B
24	4.72 ± 0.15^{a} A	2.857 ± 0.06^{a} B
30		3.012 ± 0.4^{a}

* Small letters indicate comparison of column and large letters indicate comparison of row.

LSD test, food reached the intestinal distal in T_1 and T_2, after 11.69 and 23.57 h, respectively (Fig. 2). While the distal part of food, reached after 19.81 and 29.28 h (Fig. 3).

Intestinal protein: According to Table 1, soluble protein concentrations in Caspian kutum in T_2 was lower than that of T_1 (P<0.05). The soluble protein levels increased from 0 to 1 h post feeding, decreased from 3 to 5 h post feeding and increased again from 8 to 24 h after feeding in T_1. While decreased 0 to 1 h after feeding, increased from 1to 3 h after feeding, then decreased from 5 to 8 h after feeding and increased again from 16 to 30 h after feeding in T_2.

Proteolytic enzymes: The influence of the treatment factor, time factor and their interaction on the specific activity of trypsin was significant (P<0.05). The specific trypsin activity in the experimental period is shown in Table 2. Although no significant difference was noticed, the enzymatic activity in T_1 was lower compared to T_2 (P>0.05). The peak of enzyme activity occurred at 8 and 16 h post feeding in T_1 and T_2, respectively and declined thereafter. The enzyme activity decreased from 0 to 1 and 0 to 3 h post feeding in T_1 and T_2, respectively. The effects of treatment, time and their interaction on the chymotrypsin specific activity were significant (P<0.05). Table 3 represents the specific activity of chymotrypsin during the experimental period. The activity of chymotrypsin in T_1 was lower than that of T_2. The peak of enzyme activity occurred at 5 and 8 h post feeding in T_1 and T_2, respectively and declined thereafter. Enzyme activity decreased from 0 to 1 and 0 to 3 h post feeding in T_1 and T_2, respectively.

α-Amylase: The analysis for amylase specific activity showed significant effect of treatment, time and their interaction (P<0.05). The specific activity of α-amylase during the experimental period is shown in Table 4. The enzymatic activity of α-amylase inT_1 was lower thanT_2.The peak of enzyme activity occurred at 5 and 8 h after feeding and

Table 2. Changes in specific trypsin activity (mean ± SE, n=3) post feeding in Caspian kutum reared in different water temperature (T_1: Summer and T_2: Winter).

Time/Trypsin	Treatment 1	Treatment 2
0	0.039 ± 0.003^{b} A	0.0416 ± 0.004^{b} A
1	0.021 ± 0.005^{d} A	0.0356 ± 0.004^{bc} A
3	0.024 ± 0.006^{cd} A	0.0271 ± 0.007^{c} A
5	0.031 ± 0.002^{bc} A	0.0391 ± 0.008^{bc} A
8	0.054 ± 0.002^{a} A	0.0573 ± 0.006^{a} A
16	0.036 ± 0.01^{b} B	0.0597 ± 0.003^{a} A
24	$^{x}0.019\pm0.004^{b}$ B	0.0419 ± 0.003^{b} A
30		0.038 ± 0.005^{b}

* Small letters indicate comparison of column and large letters indicate comparison of row.

Table 3. Changes in specific cymotrypsin activity (mean ± SE, n=3) post feeding in Caspian kutum reared in different water temperature (T_1: Summer and T_2: Winter).

Time/Cymotrypsin	Treatment 1	Treatment 2
0	0.81 ± 0.05^{bc} A	0.9 ± 0.04^{b} A
1	0.42 ± 0.06^{d} B	0.65 ± 0.02^{d} A
3	0.68 ± 0.05^{bcd} A	0.62 ± 0.05^{d} A
5	1.21 ± 0.49^{a} A	1.23 ± 0.05^{a} A
8	1.02 ± 0.051^{ab} B	1.30 ± 0.02^{a} A
16	0.52 ± 0.0^{cd} B	0.96 ± 0.03^{b} A
24	0.43 ± 0.02^{d} B	0.83 ± 0.03^{c} A
30		0.65 ± 0.04^{d}

* Small letters indicate comparison of column and large letters indicate comparison of row.

Table 4. Changes in specific amylase activity (mean ± SE, n=3) post feeding in Caspian kutum reared in different water temperature (T_1: Summer and T_2: Winter).

Time/ Amylase	Treatment 1	Treatment 2
0	5.75 ± 0.767^{c} A	8.418 ± 0.396^{c} A
1	7.557 ± 2.206^{b} A	$7.187\pm0.2d^{e}$ A
3	7.925 ± 2.814^{b} A	7.629 ± 0.195^{d} A
5	10.799 ± 0.485^{a} A	10.631 ± 0.309^{b} A
8	10.271 ± 0.931^{a} A	11.913 ± 0.734^{a} A
16	7.268 ± 0.625^{c} A	11.718 ± 0.242^{a} B
24	6.792 ± 0.402^{c} A	7.634 ± 0.51^{d} A
30		6.59 ± 0.401^{e}

* Small letters indicate comparison of column and large letters indicate comparison of row.

declined thereafter in T_1 and T_2, respectively.

Alkaline phosphatase: The treatment factor, time factor and their interaction had significant effect on the specific activity of alkaline phosphatase (P<0.05). Table 5 shows the alkaline phosphatase specific activity during the experiment. The enzymatic activity of alkaline phosphatase in T_1 was lower than that of T_2. The peak of enzyme activity occurred at 8 and 16 h after feeding and declined thereafter in T_1 and T_2, respectively. Enzyme activity decreased from 0 to 1 and 0 to 3 h after feeding in T_1 and T_2, respectively.

Comparison of digestive enzymes activity: The lowest alkaline phosphatase, amylase, chymotrypsin and trypsin specific activities were observed in in T_1. The amylase had the maximum enzymatic activity between all enzymes analyzed (P<0.05). In addition, among both proteases, the activity of trypsin was

Table 5. Changes in specific alkaline phosphatase activity (mean ± SE, n=3) post feeding in Caspian kutum reared in different water temperature (T_1: Summer and T_2: Winter).

Time/ Alkaline phosphatase	Treatment 1	Treatment 2
0	1.87 ± 0.4^{ab} A	2.254 ± 0.432^{b} A
1	1.43 ± 0.35^{b} A	1.746 ± 0.185^{c} A
3	2.22 ± 0.21^{ab} A	1.459 ± 0.103^{cd} A
5	$1.84\ 0.36^{ab}$ A	2.496 ± 0.462^{b} A
8	2.6 ± 0.85^{a} A	3.453 ± 0.176^{a} A
16	1.73 ± 0.05^{b} B	3.504 ± 0.182^{a} A
24	1.58 ± 0.07^{b} B	2.679 ± 0.315^{b} A
30		1.055 ± 0.077^{d}

* Small letters indicate comparison of column and large letters indicate comparison of row.

lower than that of chymotrypsin.

Discussion

Food transit time: This study demonstrates that water temperature has a significant effect on the food transit time in *R. kutum*. Factors that affect food transit time may also affect appetite and feed intake (Vinagr et al., 2007; Imsland et al., 2001). In this study higher evacuation rate of the gastrointestinal tract was observed inT_1. This is in agreement with the results of previous studies (Shimeno et al., 1993; Nakada, 2000; Watanabe et al., 2001), which water temperature has been shown to influence feed passage time and digestion rates. Also Miegel et al. (2010) reported the stomachs were almost empty in *Seriol alalandi* at the 20 h and 36 h sampling time in summer and winter, respectively. In addition, study on Japanese yellowtail (*Seriola quinqueradiata*) showed that the stomach evacuation time was less than 12 h after feeding at water temperatures between 20 and 28°C, whereas the stomach was empty less than 48 h after feeding in winter, when water temperatures was 13 and 18°C (Nakada, 2000; Watanabe et al., 2001). The gut evacuation time in *R. kutum* increased during in lower temperature due to the slower gut motility (Miegel et al., 2010).

Intestinal protein activities: In the present study, soluble proteins changes trend was different in the two treatments. The amount of soluble protein in T_2 was lower than that of T_1. This could be support the hypothesis suggested by Raoofinia (2011) that reduction of soluble proteins intestinal and tissue occurs due to stress. Stress causes an immediate mobilization of protein resources from the intestine, apparently for systemic use. Also it has been reported in other fish species that reductions of endogenous protein before the lipid reserves are completely utilized (Greene, 1926; Templeman and Andrews, 1956; Love, 1958). Therefore, the reduction of soluble protein in *R. kutum* in T_2 due to stress-induced, decrease food intake, under nutritional stress and lack of nutrients and therefore it begins to break down protein.

Digestive enzyme activities: The results revealed that tendency for protease, amylase and alkaline phosphatase activities is higher in T_2 due to slower gut motility at colder water temperatures or slower movement of digesta through the gastrointestinal tract, which allow enzymes within the digesta to accumulate (Miegel et al., 2010).

A study on the Japanese yellowtail showed that the activity of stomach enzymes, trypsin and chymotrypsin, within the pyloric caeca decreased by 10% and 30%, respectively when water temperature dropped from 25°C to 16°C (Kofuji et al., 2005). Enzyme activity could also be higher during winter due to the lower feed intake. In winter, when fish are under nutritional stress due to the reduction in feed intake, enzymes activity may increase to compensate and maximize the nutrient digestion and absorption (Miegel et al., 2010).

In marine fishes, increase of digestive enzyme

activity has been reported due to food deprivation (Lamarre et al., 2004). Also Cara et al. (2007) reported increase of trypsin and chymotrypsin activities in Sea bass (*Dicentrarchus labrax*), following food deprivation. Indeed reduced feed intake in lower temperature would be compensated by increase of enzyme activity and protein digestion capacity.

The trypsin activity was also higher at lower temperatures in *Anarhichas minor* that probably such a positive recompense mechanism renders available protein energy from food sources or internal reserves more efficiently harnessed and available for growth and physiological maintenance (Savoie et al., 2008).

Digestive enzyme activities after feeding: Food consumption is caused changes in digestive enzymes activity (Onishi et al., 1973). The study of digestive enzyme activities changes in *Chrias guriepinus* after feeding revealed gradual decrease of activity after the 4 h post feeding (Uys et al., 1987). The present study showed that peak of enzymes activities were 5 and 8 hour after feeding and after the 15 and 18 h, the activities gradually decreased. The decrease of the protease and alkaline phosphatase specific activities of *Rutilus kutum* during 0 to 1 and 0 to 3 h after feeding in T_1 and T_2, respectively was probably due to dilution by the food and an inadequate rate of secretion (Uys et al., 1987). Previous works showed that maximum trypsin activity in *Oreochromis niloticus* (Uscanga et al., 2010) and *Cyprinus carpio* (Onishi et al., 1973) occur 6 and 5 h post feeding. The peak of enzyme activity of *Rutilus kutum* was in line with those obtained previously on other Cyprinids

In conclusion, this study showed that temperature had significant impact on the food transit time and digestive enzyme activity in Caspian kutum. Thus the feeding amount and frequency should be determined considering temperature.

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