Investigation of Fatty-Acid Compositions of Female Rana Dybowskii before Hibernation

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***Abstract* - We investigate changes in fatty acid compositions in Rana dybowskii (species of hypoptychid fish found in shallow salt water off the coasts of Japan and Sakhalin) occurring before hibernation. R. dybowskii of different sizes were captured at different times in the autumn of 2011. Fatty acid contents were measured in the eggs, oviducts, and carcasses of R. dybowskii by gas chromatography-mass spectrometry. The unsaturated fatty acid contents and ratios of polyunsaturated fatty acids to fatty acids were both relatively lower in R. dybowskii with lower body weights in early autumn, compared to R. dybowskii with higher body weights. The relative unsaturated fatty acid contents and ratios of polyunsaturated fatty acids to fatty acids were both highest when hibernation began. The membrane fluidity of R. dybowskii during hibernation was maintained by increased unsaturated fatty acid contents in the body to support life during hibernation.**

Keywords - Rana dybowskii, gas chromatography-mass spectrometry (GC-MS), fatty acids

Ⅰ. INTRODUCTION

As indicated in Fauna Sinica: Amphibia (Volume 2; 2009), R. temporaria chensinensis David, which is distributed in northeast China, is identical to R. dybowskii Güenther [1], and its common name is the Chinese brown frog. R. temporaria chensinensis David was named by Pere David in 1875, according to a specimen identified in Yinjiapo, Qinling and was initially named R. chensinensis [2]. In 1940, when Pope and Boring investigated amphibians in China, they posited that R. temporaria chensinensis David was a subspecies of the European R. temporaria; thus, it was re-named as R. temporaria chensinensis [3]. In 1961, Chengzhao Liu and Shuqin Hu argued that this conclusion was oversimplified and that such classification of the relatively complex R. temporaria chensinensis taxa in China conflicted with their known characteristics. However, due to a lack of specimens and related information, they continued referring to the Chinese brown frog as R. temporaria chensinensis [4], which has been used since. In 1967, Guillemin reported that the 2n chromosome number of European frog R. temporaria is 26 [5], whereas Chinese scholars Zhengan Wu reported in 1981 and 1982 that the 2n chromosome number of Chinese brown frogs produced in Beijing and the Northeast is 24 [6,7]. Therefore, the Chinese brown frog should be considered an independent species, designated as R. chensinensis. Gang Wei and Fuguan Chen suggested there 4 subspecies of Chinese brown frogs exist, namely R. chensinensis chensinensi, R. chensinensis lanzhouensis Wei et Chen, R. chensinensis kangdingensis Wei et Chen, and R. chensinensis changbaishanensis Wei et Chen [8].

In 1999, Feng Xie, Chang-yuan Ye, and Liang Fei suggested that the northeastern population of R. chensinensis should be classified as a separate species, rather than a subspecies, and that it should be designated as R. dybowskii [9], while R. temporaria chensinensis and R. chensinensis changbaishanensis should be synonymous. This suggestion was accepted in Fauna Sinica: Amphibia (Volume 2).

R. dybowskii are mainly distributed in 3 provinces of northeast China. In addition, they are also distributed in northeast Mongolia, far east Russia, east Mongolia, North Korea, and Tsushima, Japan [1]. In the spring, R. dybowskii complete their mating, spawning, and breeding in small ponds in the hilly regions, and subsequently live in forests. R. dybowskii also live in forests after tadpole metamorphosis. In the summer, they inhabit broadleaf and mixed, coniferous forests with good vegetation conditions and generally inhabit coastal hills and mountainous regions at altitudes of approximately 1000 meters. In rainy weather during the autumn, R. dybowskii emerge from the forest and enter the mountain rivers or streams to prepare for hibernation. They begin hibernation in early winter, which lasts for nearly 6 months.

Ⅱ. MATERIALS

A. Experimental materials

R. dybowskii were captured from the Shangying Forest Management Bureau, Taoyuan Forestry Centre, Weishan Banzi Miaogou in Shulan City, Jilin Province, China (longitude: 127°25.603; latitude: 44°09.695; altitude: 1109 feet) on October 8, October 26, and November 10, 2011.

They were identified as R. dybowskii Güenther by Professor Yiping Li of the Changchun University of Chinese Medicine.

B. Reagents

Petroleum ether (30°C–60°C), potassium hydroxide, methyl alcohol, boron trifluoride diethyl etherate, and normal hexane were all of Chinese analytical grade.

C. Experimental instruments

An Agilent 7890/5975C gas chromatograph-mass spectrometer (Agilent Technologies), a DB-5MS (30 m × 0.25 mm × 0.25 μm) quartz capillary column, a constant-temperature water bath (Jiangsu Changshu Medical Instrument Factory), and a BP211D electronic analytical balance (Sartorius Co., Germany) were used for experiments conducted in this study.

Ⅲ. METHODS AND RESULTS

A. Sample preparation

R. dybowskii were sacrificed in hot water (80°C) and individually weighed. Subsequently, they were dried at a temperature of 20 ± 2°C with a relative humidity of 40–45%. After drying, the oviducts, eggs, and carcasses (full body with all internal organs removed) were dried at 40°C to a constant weight, after which dry oviduct, egg, and carcass samples were obtained.

B. Fatty acid methyl ester

Five-gram dry oviduct samples were placed in 50-ml stoppered conical flasks. Next, 25 ml of petroleum ether (30°C–60°C, boiling-point range) was added, and the resulting solution was mixed for 10 min. The samples were left overnight and filtered to remove the solvent on the following day. Next, 0.1 g of extracted oil from the samples was added to a small, stoppered-glass tube containing 3 ml of 0.5 mol/L potassium hydroxide-methanol solution, and the sample was placed into a 60°C water bath for 30 min. After cooling, 2 ml of 30% boron trifluoride diethyl etherate-methanol solution (2: 1) was added, and the sample was heated on a 60°C water bath for 2 min. After cooling to room temperature, 3 ml distilled water and 2 ml normal hexane were added and vortexed. One milliliter of supernatant was transferred to a 10-ml volumetric flask and diluted to the mark using normal hexane. The sample was then filtered with 0.45-μm microporous membrane, and the filtrate was collected for gas chromatography-mass spectrometry (GC-MS) analysis, as described [10].

An appropriate amount of eggs was weighed out and placed in a 100-ml stoppered, conical flask. Fifty milliliters of petroleum ether (30°C–60°C, boiling-point range) was added, and the sample was mixed for 10 min. The sample was left overnight and filtered to remove the solvent on the following day. Next, 0.1 g of extracted oil was added to a small, stoppered glass tube, 3 ml of 0.5 mol/L potassium hydroxide-methanol solution was added, and the samples was placed in a 60°C water bath for 30 min. After cooling, 2 ml 30% boron trifluoride diethyl etherate-methanol solution (2: 1) was added and the sample was placed in a 60°C water bath for 2 min. After cooling to room temperature, 3 ml distilled water and 2 ml normal hexane were added, and the sample was vortexed. One milliliter of supernatant was transferred to a 10-ml volumetric flask and diluted to the mark using normal hexane. The sample was filtered with a 0.45-μm microporous membrane, and the filtrate was collected for GC-MS analysis.

An appropriate amount of carcass was placed in a stoppered 100-ml conical flask. Fifty milliliters of petroleum ether (30°C–60°C, boiling-range range) was added and the sample was mixed for 10 min. The sample was left overnight and filtered to remove the solvent. Next, 0.1 g of extracted oil was transferred to a small stoppered glass tube, 3 ml of 0.5 mol/L potassium hydroxide-methanol solution was added, and the sample was placed into a 60°C water bath for 30 min. After cooling, 2 ml 30% boron trifluoride diethyl etherate-methanol solution (2: 1) was added, and the sample was placed in a 60°C water bath for 2 min. After cooling to room temperature, 3 ml distilled water and 2 ml normal hexane were added, and the sample was vortexed. One milliliter of supernatant was transferred to a 10-ml volumetric flask and diluted to the mark using normal hexane. The sample was then filtered with a 0.45-μm microporous membrane, and the filtrate was collected for GC-MS analysis.

C. Combined GC-MS conditions

GC-MS experiments were performed as follows. A quartz capillary DB-5MS chromatography column (30 m × 0.25 mm × 0.25 μm) was used with a pre-column pressure of 80 Kpa and a column temperature of 50°C–270°C. The column was heated from 50°C–200°C at a rate of 20°C/min and from 200–270°C at 5°C/min. The temperature of vaporizer chamber and inlet temperature was 270°C. High-purity He (≥99.9%) was used as the carrier gas. The flow rate of the carrier gas was 40 ml/min, and the sample volume was 1 μL. An electron ionization (EI) ion source was used for MS experiments with an ion-source temperature of 250°C, an electron energy of 70 electron volts, a mass/change (m/z) scan range of 35–350 atomic mass units, and a volume delay time of 3 min.

Ⅳ. RESULTS

R. dybowskii oviduct, egg, and carcass samples with different capture times and weights were processed and analyzed according to above conditions. The chromatographic peaks of mass spectra obtained from the tests were identified by searching through MS data in a standard spectral-library workstation. The MS were identified as: 1: methyl tridecanoate, m/z: 228 (M+), 185, 143, 87, and 74 (base peaks); 2: methyl myristate, m/z: 242 (M+), 199, 185, 143, 129, 87, 74 (base peaks), and 43; 3 methyl pentadecanoate, m/z: 256 (M+), 225, 213, 143, 74 (base peaks), and 43; 4: methyl palmitoleate, m/z: ; 5: methyl palmitate (hexadecanoate), m/z: 270 (M+), 227, 199, 143, 97, 87, 74 (base peaks), and 43; 6: methyl linoleate, m/z: 294 (M+), 262, 222, 180, 95, 67, and 59 (base peaks); 7 methyl linolenate, m/z: 292 (M+), 262, 222, 180, 95, 67, and 59 (base peaks); 8: methyl oleate, m/z: 296 (M+), 264, 222, 180, 97, 74, and 59 (base peaks); 9: methyl stearate, m/z: 298 (M+), 255, 213, 199, 143, 97, 74 (base peaks), and 43; 10: methyl arachidonate, m/z: 302 (M+), 207, 160, 91, and 79 (base peaks); and 11: methyl eicosapentaenoate (EPA), m/z: 316 (M+), 220, 207, 176, 91, and 79 (base peaks).

Eleven fatty acids were detected: tridecanoic acid, myristic acid, pentadecanoic acid, palmitoleic acid, palmitic acid (hexadecanoic acid), linoleic acid, linolenic acid, oleic acid, stearic acid, arachidonic acid, and EPA, which included 5 saturated fatty acids and 6 unsaturated fatty acids. The GC-MS total-ion current chromatogram peak areas were normalized, and the relative percentage compositions of the fatty acids were obtained. The results are shown in the tables and figures below.

TABLE 1: FATTY ACIDS STUDIED AND THEIR CONTENTS IN THE OVIDUCTS OF *R. DYBOWSKII* WITH DIFFERENT CAPTURE TIMES AND BODY WEIGHTS

Peak No	Fatty acids	Relative percentage content of fatty acids [%]								
		Capture date：October 8, 2011			Capture date：October 26, 2011			Capture date：November 10, 2011		
		Weight: 25.86 ± 2.81 [g]	Weight: 35.21 ± 2.79 [g]	Weight: 45.42 ± 2.83 [g]	Weight: 25.38 ± 2.78 [g]	Weight: 35.23 ± 2.79 [g]	Weight: 45.36 ± 2.71 [g]	Weight: 25.29 ± 2.86 [g]	Weight: 35.12 ± 2.83 [g]	Weight: 45.59 ± 2.82 [g]
1	Methyl tridecanoate	0.781	0.763	0.824	0.721	0.746	0.801	0.756	0.728	0.812
2	Methyl myristate	3.201	3.519	3.251	2.586	2.331	2.382	2.144	2.217	2.251
3	Methyl pentadecanoate	1.578	1.536	1.438	1.604	1.583	1.603	1.809	1.537	1.583
4	Methyl palmitoleate	4.166	5.735	5.351	4.127	4.893	5.47	5.94	5.496	7.127
5	Methyl palmitate	17.603	18.107	18.436	17.024	18.044	18.135	18.326	18.441	18.525
6	Methyl linoleate	10.061	10.843	11.34	9.582	12.621	12.51	9.324	11.308	11.041
7	Methyl linolenate	9.889	10.984	10.047	10.152	11.586	12.228	10.747	13.37	13.855
8	Methyl oleate	27.109	27.873	28.598	31.195	33.823	33.866	31.697	32.428	33.282
9	Methyl stearate	3.544	3.912	3.813	3.968	3.713	3.845	3.963	3.820	3.809
10	Methyl arachidonate	2.148	2.531	2.618	2.567	2.893	2.915	2.536	2.943	2.934
11	Methyl eicosapentaenoate	1.989	2.275	2.412	2.304	2.729	2.762	2.413	2.626	2.887
Proportion of unsaturated fatty acid content of total fatty acid content [%]		67.46	68.39	68.5	69.82	72.18	72.27	69.88	71.82	72.5
Ratio of polyunsaturated fatty acid to saturated fatty acid content [p/s]		0.9	0.96	0.95	0.95	1.12	1.13	0.93	1.13	1.14

TABLE 2: FATTY ACIDS STUDIED AND THEIR CONTENTS IN THE EGGS OF *R. DYBOWSKII* WITH DIFFERENT CAPTURE TIMES AND BODY WEIGHTS

Peak No	Fatty acids	Relative percentage content of fatty acids [%]								
		Capture date: October 8, 2011			Capture date: October 26, 2011			Capture date: November 10, 2011		
		Weight: 25.86 ± 2.81 [g]	Weight: 35.21 ± 2.79 [g]	Weight: 45.42 ± 2.83 [g]	Weight: 25.38 ± 2.78 [g]	Weight: 35.23 ± 2.79 [g]	Weight: 45.36 ± 2.71 [g]	Weight: 25.29 ± 2.86 [g]	Weight: 35.12 ± 2.83 [g]	Weight: 45.59 ± 2.82 [g]
1	Methyl tridecanoate	0.783	0.721	0.719	0.804	0.829	0.841	0.819	0.854	0.863
2	Methyl myristate	3.856	3.688	3.582	3.852	3.436	3.055	3.202	3.616	3.17
3	Methyl pentadecanoate	1.519	1.538	1.602	1.528	1.569	1.615	1.541	1.578	1.601
4	Methyl palmitoleate	5.302	6.228	5.629	5.585	5.717	5.196	5.259	5.074	5.574
5	Methyl palmitate	18.571	18.102	18.643	18.506	17.735	17.276	18.43	18.243	18.897
6	Methyl linoleate	8.521	8.031	8.018	8.175	10.152	10.433	9.075	10.532	10.447
7	Methyl linolenate	8.613	9.752	9.444	10.11	10.415	11.537	10.123	11.013	12.27
8	Methyl oleate	25.828	25.848	26.991	28.894	28.805	28.343	27.734	29.256	29.865
9	Methyl stearate	4.545	4.873	4.974	4.672	4.993	4.844	4.575	5.067	5.782
10	Methyl arachidonate	1.479	1.557	2.06	1.131	1.284	1.264	1.355	1.477	1.454
11	Methyl eicosapentaenoate	1.305	1.357	1.876	1.052	1.081	1.132	1.057	1.203	1.331
Proportion of unsaturated fatty acid content of total fatty acid content [%]		62.55	64.59	64.66	64.21	66.79	67.69	63.65	66.6	66.78
Ratio of polyunsaturated fatty acid to saturated fatty acid content [p/s]		0.68	0.71	0.72	0.69	0.8	0.88	0.76	0.83	0.84

TABLE 3: FATTY ACIDS STUDIED AND THEIR CONTENTS IN THE CARCASSES OF *R. DYBOWSKII* WITH DIFFERENT CAPTURE TIMES AND BODY WEIGHTS

Peak No	Fatty acids	Relative percentage content of fatty acids [%]								
		Capture date: October 8, 2011			Capture date: October 8, 2011			Capture date: October 8, 2011		
		Weight: 25.86 ± 2.81 [g]	Weight: 35.21 ± 2.79 [g]	Weight: 45.42 ± 2.83 [g]	Weight: 25.38 ± 2.78 [g]	Weight: 35.23 ± 2.79 [g]	Weight: 45.36 ± 2.71 [g]	Weight: 25.29 ± 2.86 [g]	Weight: 35.12 ± 2.83 [g]	Weight: 45.59 ± 2.82 [g]
1	Methyl tridecanoate	0.612	0.635	0.691	0.634	0.671	0.662	0.628	0.601	0.682
2	Methyl myristate	3.762	3.652	3.061	3.094	3.052	3.667	3.867	3.112	3.176
3	Methyl pentadecanoate	1.463	1.481	1.472	1.521	1.534	1.568	1.624	1.598	1.639

4	Methyl palmitoleate	5.546	5.676	5.763	5.199	5.565	5.256	5.391	5.673	5.837
5	Methyl palmitate	18.905	18.07	18.977	18.748	18.448	18.615	18.906	18.455	18.445
6	Methyl linoleate	8.505	8.02	8.849	10.736	10.115	10.928	8.095	10.372	10.677
7	Methyl linolenate	9.373	7.715	8.264	9.061	10.289	8.381	10.211	9.619	9.877
8	Methyl oleate	21.241	21.735	21.956	22.261	22.513	23.834	23.58	23.352	22.85
9	Methyl stearate	4.874	4.947	4.569	5.736	5.365	5.262	5.673	5.72	5.418
10	Methyl arachidonate	2.561	2.562	2.699	2.307	2.147	2.344	2.861	2.318	2.678
11	Methyl eicosapentaenoate	2.718	2.738	2.558	2.197	2.918	2.164	2.256	2.901	2.535
Proportion of unsaturated fatty acid content of total fatty acid content [%]		62.78	62.73	63.51	63.51	64.81	63.98	63.06	64.78	64.97
Ratio of polyunsaturated fatty acid to saturated fatty acid content [p/s]		0.78	0.73	0.78	0.81	0.87	0.8	0.76	0.85	0.88

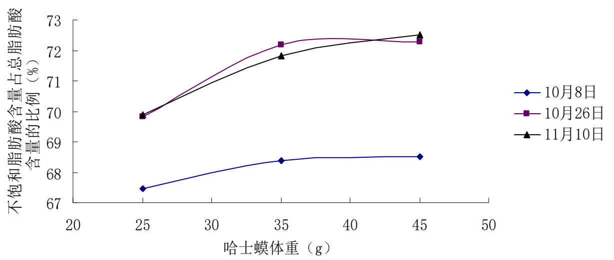

Fig. (1). Proportion of unsaturated fatty acids in the oviducts of R. dybowskii with different capture dates and body weights. The y-axis shows the ratio of unsaturated fatty acid contents to the total fatty acid content on a percentage basis. The x-axis shows the body weights of R. dybowskii. The legend shows the capture dates: October 8, October 26, and November 10, 2011.

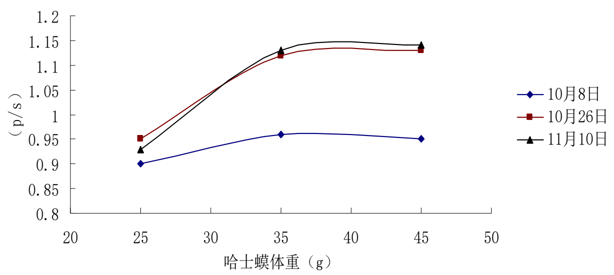

Fig. (2). P/s ratios in the oviducts of R. dybowskii with different capture dates and body weights. The X-axis shows the body weights of the R. dybowskii specimens. The legend shows the capture dates: October 8, October 26, and November 10, 2011.

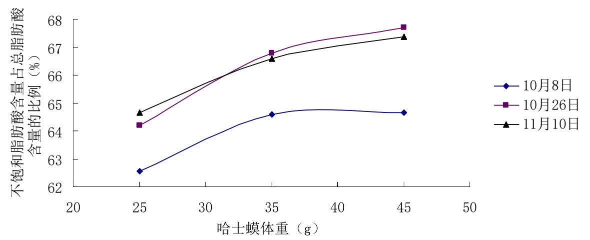

Fig. (3). Proportion of fatty acids in the eggs of R. dybowskii with different capture times and body weights. The y-axis shows the proportion of unsaturated versus fatty acid contents (%). The x-axis shows the body weights of the R. dybowskii. The legend shows the capture dates: October 8, October 26, and November 10, 2011.

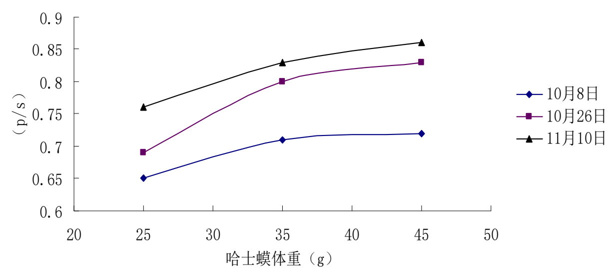

Fig. (4). P/s ratios in the oviducts of R. dybowskii of different capture dates and body weights. The X-axis shows the body weights of the R. dybowskii specimens. The legend shows the capture dates: October 8, October 26, and November 10, 2011.

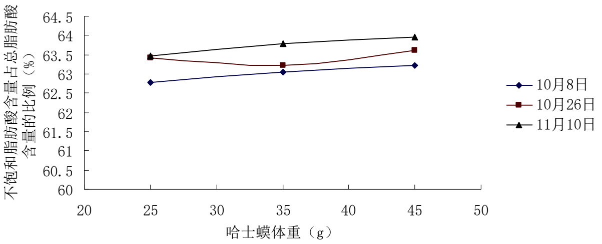

Fig. (5). Proportion of unsaturated fatty acids in the carcasses of R. dybowskii specimens with different capture dates and body weights. The y-axis shows the proportion of unsaturated fatty acid contents relative to the total fatty acid content (%). The X-axis shows the body weights of the R. dybowskii specimens. The legend shows the capture dates: October 8, October 26, and November 10, 2011.

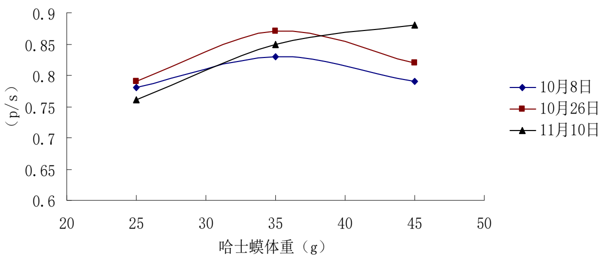

Fig. (6). P/s ratios of R. dybowskii carcasses with different capture dates and body weights. The X-axis shows the body weights. The capture dates were October 8, October 26, and November 10, 2011.

V. DISCUSSION

R. dybowskii generally travel from forests to mountain streams in mid-to-late September and usually begin hibernation in November. Our experimental results showed that R. dybowskii begin hibernation near November, during which time the unsaturated fatty acid content, the proportion of unsaturated versus total fatty acid contents, and the p/s ratios were the highest in their oviducts, eggs, and carcasses. The average weight of a 2-year-old female R. dybowskii was approximately 20 g, indicating that it can reproduce by laying eggs before reaching adulthood. Three-year-old R. dybowskii are adults and generally have body weights exceeding 30 g. The experimental results presented in this study indicate that the unsaturated fatty acid contents, the proportion of unsaturated versus total fatty acid contents, and the p/s ratios were all lower in the oviducts, eggs, and carcasses of smaller-sized (~20 g) R. dybowskii,. The unsaturated fatty acid contents, the proportion of unsaturated versus total fatty acid contents, and p/s ratios were lower in R. dybowskii carcasses than in their oviducts and eggs.

Polyunsaturated fatty acids play an important role in increasing membrane fluidities in animals. The degree of unsaturation of membrane lipids can be regulated by alterations in the activity of fatty acid desaturase, which increases the ability of organisms to resist cold temperatures. Long-chain unsaturated fatty acids, such as EPA and docosahexaenoic acid, are essential components lipid membranes and can maintain cell membrane fluidity and permeability. During hibernation, R. dybowskii exist under conditions of low temperature, low metabolism, and a low heart rate. In addition, their blood flow slows down, while cell membrane fluidity depends on membrane compositions and body temperatures. Thus, R. dybowskii maintain membrane fluidity by increasing the long-chain unsaturated fatty acids compositions within their bodies to sustain life during hibernation.

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