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Discovery of Fenthion Poisoning in Two Japanese Cranes (*Grus japonensis*) Found Dead in Hokkaido, Japan

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Hokkaido is a large island located in northern Japan. Farmland is prevalent in eastern Hokkaido; Kushiro-shitsugen (43°09’ N, 144°26’ E), registered for the Ramsar convention on wetlands, is also located in this region. The Japanese crane (*Grus japonensis*, protected by the Government) is the biggest bird in Japan, lives only in eastern Hokkaido, and is known to breed on wetlands in the summer. Cranes instinctively possess a considerable degree of wariness, but their ecology has been strongly compromised by recent changes in the external environment. There are two types of changes. One is the decrease in wetlands; another is habituation to feeding by humans. In fact, they now nest near houses, and have been known to enter a cattle barn for feeding. The population density of this region is relatively low; therefore, it is thought that the region is independent of unintentional by-products based on industrial activities. However, for the cranes, we think that increased contact with intentional chemicals like herbicides and insecticides has become a larger risk. In addition, not only “the relationship between feed and birds” but also “the relationship between birds and their predators” have been indicated as secondary poisoning hazards (Comber et al. 2003; Berny et al. 1997; Hunt et al. 1991).

The most common poisons in avian species are heavy metals, botulism, and agricultural chemicals. For example, lead is one of the main causes of poisoning through direct or indirect exposure by sinkers and lead shot (Johansen et al. 2004; de Francisco et al. 2003); if fewer immunized birds are in wetlands, they may be affected strongly with botulism (Rocke et al. 2000; Wobeser 1997). From the point of view of the sensitivity to avian species, organophosphorus compounds, found in various agricultural chemicals, are the most hazardous because they are designed to produce acute cholinergic effects through inhibition of acetylcholinesterase (Richardson 1995). In fact, the comparison of lethal dose 50 (LD_{50}) between chicken and rats leads to the conclusion that most of the organophosphorus compounds delivering intermediate syndromes to rats tend to produce more serious damage in chickens (Hayes and Laws 1991).

In October 2002, a pair of Japanese crane carcasses was found in a wheat field along a riverside of Memanbetsu town in Hokkaido, Japan (Figure 1). An autopsy

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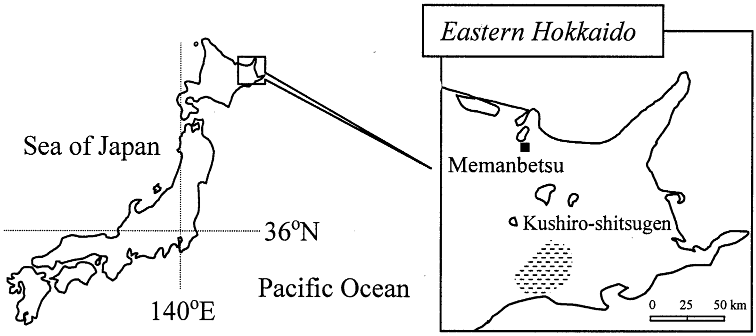

Figure 1. Map of Japan showing Memanbetsu.

and pathological inspection prompted us to carry out an investigation on agricultural chemicals used around the area. To clarify the cause of their death, we analyzed three sites (breast muscles, livers, and stomach residues) in the cranes.

In this study, gas chromatography-mass spectrometry (GC-MS) operating in scan mode notably revealed the agricultural chemical fenthion in the crane stomachs. The concentrations in the two Japanese cranes were determined by GC-MS; further, the concentrations of several metals were also measured by inductively coupled plasma mass spectrometry (ICP-MS) to evaluate the possibility of heavy metal poisoning. As far as we know, this is the first publication to identify the exact agricultural chemical responsible for a case of acute poisoning of this precious Japanese avian species.

MATERIALS AND METHODS

Solvents for dioxin-analysis grade (Wako Pure Chemical Industries, Osaka, Japan) were employed. Determination of three compounds (pendimethalin, trifluralin, and fenthion) was carried out by the absolute calibration method. Authentic standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and Kanto Kagaku (Tokyo, Japan). All the other chemicals (Wako Pure Chemical Industries, Osaka, Japan) were of highest grade available and were used without further purification. Purified water was obtained by passage through a Milli-Q® water purification system (Gradient A10, Nihon Millipore, Tokyo, Japan).

After determination of biological parameters, carcasses were stored at –20 °C; the autopsy was carried out at Kushiro Zoo, Kushiro city. Five grams (wet weight basis) of the site (breast muscles, livers, and stomach residues) was transferred into a glass container. Thirty milliliters of acetone/water (7:3; v/v) was added to the container; after the sample was homogenized, the homogenate was centrifuged (1500 rpm, 5 min). This procedure was repeated twice. The supernatant liquid was recovered and was reduced in a rotary evaporator. The concentrate was transferred into a separatory funnel. Twenty milliliters of sodium chloride solution (15%; w/w) and 20 ml of ethyl acetate/hexane (1:1; v/v) were added to the funnel, and the mixture was shaken. After the phases were allowed to separate, sodium sulfate anhydrous dehydrated the organic phase. Further, the extraction was carried out by addition of 10 ml of ethyl acetate/hexane (1:1; v/v) to the residue again. The organic phase was concentrated as a final extract. For fenthion, 2 ml of acetone/hexane (1:1; v/v) was substituted for the extract. A silica gel cartridge (Sep-Pak® Plus, 690 mg, Nihon Waters, Tokyo, Japan) which was washed with 10 ml of acetone/hexane (1:1; v/v) beforehand, was applied to the cleanup. After the sample was loaded, 10 ml of acetone/hexane (1:1; v/v) was used for elution of fenthion. The eluate was transferred to a centrifuge tube and dried with a gentle stream of dry nitrogen. Final concentrate dissolved in 2 ml of acetone/hexane (1:4; v/v). On the other hand, for pendimethalin and trifluralin, 2 ml of acetone/hexane (2:3; v/v) was substituted for the final extract. The sample was cleaned with a pre-washed florisil cartridge (Sep-Pak® Plus, 910 mg, Nihon Waters, Tokyo, Japan). Pendimethalin and trifluralin were eluated by using 10 ml of acetone/hexane (2:3; v/v). The eluate was transferred to a centrifuge tube and dried with a gentle stream of dry nitrogen. Final concentrate dissolved in 2 ml of acetone/hexane (1:4; v/v). The three compounds were determined by a JMS-700 (JEOL, Tokyo, Japan) mass spectrometer equipped with a 5890 series gas chromatograph (Agilent Technologies, DE, USA). DB-5MS (Varian, Walnut Creek, CA, USA: 30 m length, 0.25 mm i.d., 0.25 μm film thickness) was selected as a fused silica capillary column. Helium was employed as a carrier gas at the flow rate of 1 ml min^{-1}. One microliter of a final concentrate was injected under a splitless mode. Temperatures of an injector port and a transfer line in gas chromatograph were maintained at 280 °C and at 280 °C, respectively. The column temperature was maintained at 50 °C for 1 min, ramped to 280 °C at a rate of 10 °C min^{-1}. Ionization current, ionization, and accelerating voltage were set to 300 μA, 70 eV, and 8 kV, respectively. The resolution (R) of the mass spectrometer was maintained at R = 1000 (10% valley). Ion source temperature was held at 200 °C. The mass spectrometer was operated on the basis of selected ion monitoring (SIM) or scan mode.

For determination of metals, the following procedure was carried out. The sample (0.5 g) was decomposed with nitric acid at 140 °C for 6 hours and was concentrated on a hot plate (140 °C). The final solution for ICP-MS was prepared by dilution of the decomposition sample. HP 4500 series (Agilent Technologies, DE, USA) was used for the determination based on ICP-MS: RF output (1330 W), flow rate of carrier argon gas (1.06 l min^{-1}), sampling depth (7 mm), and s/c temperature (2 °C).

RESULTS AND DISCUSSION

Measurements of lipid weight for breast muscles showed 1.9% in the male and 1.6% in the female; livers were estimated to have 3.8% (male) and 2.1% (female). However, for the comparison with residues in stomach, concentrations were calculated on the basis of wet weight. Obvious grains of sand in stomachs were removed before pesticide analysis. Results of the autopsy were as follows: both

Table 1. Concentrations ($\mu g\ kg^{-1}$; wet wt.) of dinitroanilines in the crane sites.

Compound	Male			Female		
	muscle	liver	stomach	muscle	liver	stomach
Pendimethalin	nd	13	19	nd	191	8.5
Trifluralin	2.5	0.2	5.9	4.0	0.2	4.3

nd: not detected; stomach: stomach residues.

Japanese cranes (weight: male, 8.3 kg; female, 8.2 kg) died without injury or outside parasites. Their nutrition status was good, whereas "congestion of major organs" and "relaxation of the duodenum", and "fusion in the pancreas" were confirmed. Further, the pathological inspection indicated acute pancreatic necrosis or acute inflammation of the small intestine as a direct cause of death. These results led us to assume that the consumption of something toxic had caused death in the cranes. Considering the locations and the season, we first suspected pendimethalin and trifluralin, because both of these dinitroanilines are used as herbicides in the east of Hokkaido. Analysis was carried out in the breast muscles, livers, and residues of stomachs. Table 1 summarizes all of the data concerning pendimethalin and trifluralin. Concentrations of the two compounds were found in the range of nd–190 $\mu g\ kg^{-1}$ (pendimethalin) and in the range of 0.2–5.9 $\mu g\ kg^{-1}$ (trifluralin). However, in these compounds, the concentrations corresponding to residues in stomachs were not extremely high compared with those in the other organs; that is, we concluded that pendimethalin and trifluralin were minor contributors to the cranes' deaths at most.

On the other hand, a notable peak was detected in the organophosphorus fraction as a result of operating GC-MS in scan mode. Figure 2 (a) shows the total ion chromatogram obtained from residues in the male crane stomach. In this chromatogram, one can observe a dominant peak at 18.9 min. The mass spectrum peak is illustrated in Figure 2 (b); it was considered that *m/z* 278 was a molecular ion whereas weak fragment ions such as *m/z* 125 and *m/z* 109 were also found. For the identification of this peak, the spectrum was compared with that of National Institute of Standards and Technology (NIST) mass spectral library. It was discovered from Figure 2 (c) to correspond with the mass spectrum of fenthion. In fact, an injection of the standard solution of fenthion in GC-MS produced an identical mass spectrum at the identical retention time. Therefore, we identified the peak with fenthion, and the concentration in each organ was determined using SIM mode. The results are listed in Table 2. Fenthion was present in all the samples. The concentrations ranged widely, from 0.03 $mg\ kg^{-1}$ to 920 $mg\ kg^{-1}$; the overall concentration of fenthion was more than 1000 times as high as those of pendimethalin and trifluralin. In particular, the concentrations (approximate to 1000 $mg\ kg^{-1}$) of fenthion in stomach residues were over 1000 times higher than those (< 1 $mg\ kg^{-1}$) of the other organs. This characteristic was independent of sex; therefore, we estimated that the two cranes died immediately and simultaneously without time for excretion after the intake of something containing fenthion. The fact that the other organs also possessed over 0.01 $mg\ kg^{-1}$

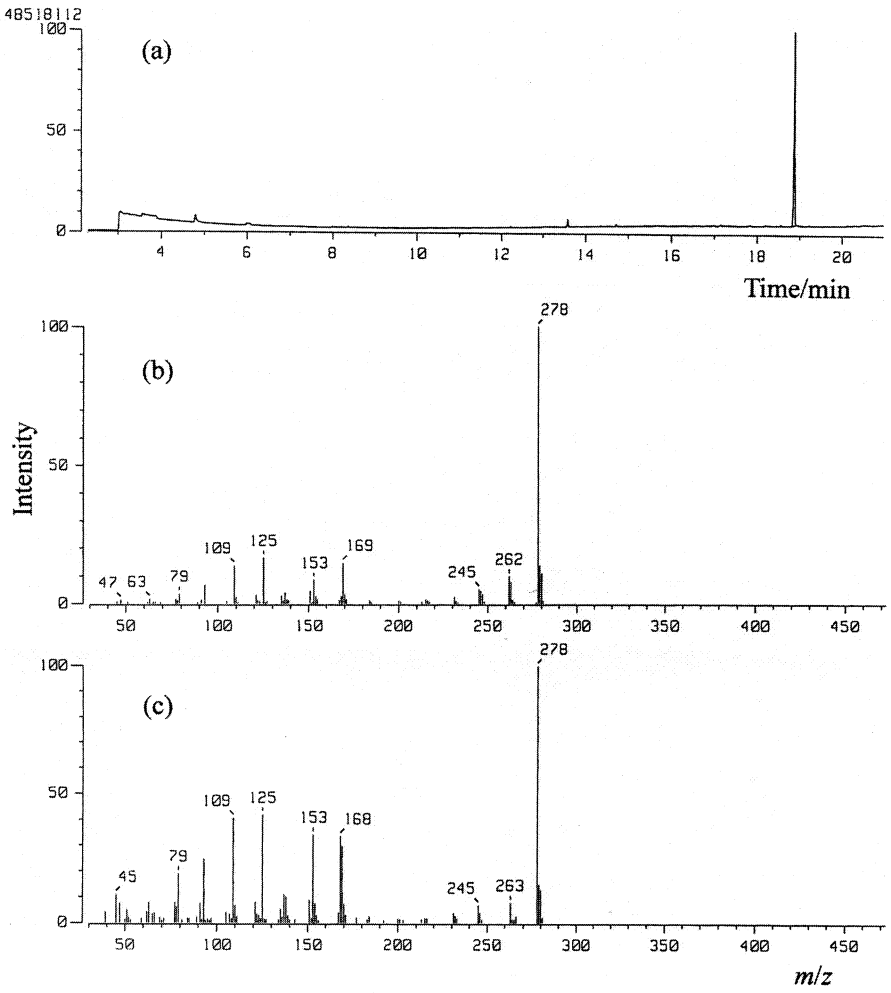

Figure 2. Identification of the notable peak in stomach residues of the male crane. (a): total ion chromatogram; (b): mass spectrum of the peak at 18.9 min; (c): mass spectrum of fenthion (NIST).

Table 2. Concentrations (mg kg^{-1}; wet wt.) of fenthion in the crane sites.

Compound	Male			Female		
	muscle	liver	stomach	muscle	liver	stomach
Fenthion	0.2	0.07	920	0.6	0.03	790

stomach: stomach residues.

Table 3. Concentrations (mg kg^{-1}; wet wt.) of metals in the crane sites.

Element	Male			Female			Pintail (F)
	muscle	liver	stomach	muscle	liver	stomach	muscle
Selenium	0.51	2.8	0.69	0.30	2.1	0.66	0.80
Lead	0.016	0.065	1.0	0.018	0.049	1.3	0.049
Arsenic	0.020	0.029	0.76	0.036	0.029	0.61	0.014
Cadmium	0.0055	0.054	0.28	nd	0.043	0.20	0.0033

nd: not detected; stomach: stomach residues; (F): adult female.

of this compound may indicate that part of the fenthion was absorbed in digestive tract and transported to other tissues. Blood was not extracted from the tissues.

Fenthion tends to have a strong toxicity for birds; for example, the LD_{50} for rodents were in the range of 150–615 mg kg^{-1} (Francis and Barnes 1963) while that of birds was less than 20 mg kg^{-1} (Hudson et al. 1984). This organophosphorus compound has been widely distributed in Africa to control birds that eat cereal crops (Keith et al. 1994). Several kinds of birds possess a sensitive response (LD_{50}: < 10 mg kg^{-1}) to the compound as follows: 2.5 mg kg^{-1} for mourning dove (*Zenaida macroura*), 4.0 mg kg^{-1} for bobwhite quail (*Colinus virginianus*), 4.63 mg kg^{-1} for rock dove (*Columba livia*), and 5.94 mg kg^{-1} for mallard duck (*Anas platyrhynchos*) (Hudson et al. 1984). If the LD_{50} of the Japanese crane is estimated based on these data, the crane may die from intake of 20–200 mg kg^{-1}. Moreover, considering the residue level (790–920 mg kg^{-1}) and the weight (male: 58.5 g and female: 70 g) of residues in stomachs, we concluded that each crane had consumed approximately 54.5 mg of fenthion. If almost all the fenthion intake remained into the stomach, the intake per 1 kg of weight becomes 6.7 mg. This is a concentration level equal to the LD_{50} of the avian species mentioned above.

Concentrations of four metals (selenium, lead, arsenic, and cadmium) were also measured to evaluate the influence of heavy metal poisoning. During spring 1989, the lead concentrations in livers (n = 15) of whooper swans (*Cygnus cygnus*) which died at Lake Miyajima (Hokkaido, Japan) from subacute lead poisoning, ranged from 5.5 to 44.3 mg kg^{-1} (Ochiai et al. 1992). Table 3 indicates that the present results (0.065 mg kg^{-1} and 0.049 mg kg^{-1}) differed from the concentration levels causing lead poisoning. The concentrations of the other metals were similar to that of the pintail that was caught at Saitama *Kamoba* (non-hunting area; Saitama Imperial Wild Duck Preserve). Although we can hardly perform a simple comparison of the concentrations for different avian species, it was clear that the concentrations of heavy metals in the two cranes were not high. The two Japanese cranes showed no exceptional concentration of typical hazardous metals.

In Japan, fenthion has been registered as a pesticide in 1960; over twenty commercially available chemicals containing the compound have been heavily used for mothproofing to beans and potatoes in Hokkaido; the use in the area reaches approximately 38000 kg per year. It must be noted that fenthion is used for not specific avian species but insects. The autopsy and the pathological inspection indicated that the intake of something toxic caused chemical poisoning in the two cranes; further, the following items were found as main contents of stomachs: rice, wheat, dragonflies, shells, vegetable fibers, gravel, and a bit of glass. Particularly strange items were not outwardly present in the stomachs; however, there was no conclusive proof that fenthion was absent. Judging from the detected concentrations and the form of commercial fenthion chemicals (oils, granule, and emulsion, for example), it may safely be assumed that the compound has already been dispersed in contents of stomachs. Diluted chemicals are usually used in order to sprinkle over crops; therefore, it would be untrue to say that the death of the two cranes was provided by the intake of commercial crops cultivated in plowed fields. It is possible to build up two hypotheses concerning the death; we lay special emphasis on stored chemicals and illegal abandonment. In the season that the dead cranes were discovered, fenthion has not been used. If the storage of agricultural chemicals is securely controlled, the former can be explained by habituation of the cranes to human beings. Namely, in a farmstead, the cranes fed on the food items which the fenthion soaked through. On the other hand, the latter indicates that the cranes swallowed directly the food items (rice, wheat, or a granular chemical itself) contaminated intentionally by fenthion. The true reason why the cranes were given a large quantity of fenthion is unclear; however, on the basis of the agricultural information in the area, we think that the possibility of executing our hypotheses is considerably high.

The fenthion concentrations of stomach residues were far higher than that of the other organs; further, the estimated level of fenthion intake was similar to the LD_{50} of other birds. Therefore, we conclude that the acute poisoning in the cranes was caused by direct fenthion consumption.

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