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JAPANESE USE OF BENI-TENGU-DAKE (*AMANITA MUSCARIA*) AND THE EFFICACY OF TRADITIONAL DETOXIFICATION METHODS

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DEDICATION

To my parents...

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ABSTRACT OF THE THESIS

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Professor Bradley C. Bennett, Major Professor

The poisonous fruiting bodies of *Amanita muscaria* (L. ex Fr.) Pers. ex Hook. are harvested by rural inhabitants of Sanada Town, Japan. These mountain villagers consume beni-tengu-take as a local delicacy, despite its potential hallucinogenic effects. The Japanese use several methods to detoxify beni-tengu-take, but believe pickling the mushrooms to be the safest. Other methods of preparation include grilling and drying the mushrooms. I documented the preparation and consumption of each detoxification method through local interviews with Japanese informants. I then used ion-interaction rp-HPLC to quantify the hallucinogenic compounds, ibotenic acid and muscimol, and determined the efficacy of each traditional detoxification method. Fresh mushrooms contained 6.17mmol/kg of ibotenic acid (LD_{50} in mice is 0.9 mmol/kg when administered orally) and 0.93mmol/kg of muscimol (LD_{50} in mice is 0.4 mmol/kg when administered orally). Grilling and drying increased the toxicity of the mushrooms. The pickling process removed all detectable amounts of both hallucinogenic compounds.

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CHAPTER I. INTRODUCTION AND BACKGROUND

Mushrooms are valued sources of food and medicine throughout the world (Cooke 1977, Hobbs 1986, Imazeki and Honda 1984). Several cultures cherish the secondary compounds from mushrooms and the mind-altering affects these chemicals produce (Furst 1972, Gartz 1996, Haard 1977, McDonald 1980, Ott 1978, Schultes and Hoffmann 1979, Tyler 1979). *Amanita muscaria* (L. ex Fr.) Pers. ex Hook. (Amanitaceae) is one such mushroom, well known for its religious uses in India, Siberia, Northwest America, and Mexico (Lowy 1972, 1974; Saar 1991a,b; Wasson 1968, 1979). In Sanada Town of Nagano Prefecture, Japan, rural people cherish these mushrooms as food and have found several methods to remove the toxic compounds. Wasson (1973) briefly described the enthusiasm with which these people collect and eat these mushrooms.

Amanita muscaria, known as beni-tengu-take (Imazeki 1973), is entwined with Japan's rich mythology (Whelan 1994). Tengu refers to elusive, magical, mountain-dwelling goblins that are respected as temple guardians and feared for their fondness of mischievous pranks (Casal 1957). Rural inhabitants of Japan believe tengu take a modified human form with red faces, long noses, glittering eyes, and wings (Piggott 1987). These goblins also were believed temporarily to possess Buddhist monks (yamabushi) who lived deep in the montane forests (Earhart 1970). In de Visser's review (1908) of a Japanese folk-story, entitled *Mottomo no Soshi,* tengu became, "drunk by eating a certain kind of mushroom," presumably *Amanita muscaria*. Beni-tengu-take's bioactive compounds could easily account for such a response. Yet, people in Sanada

Town consume these mushrooms as a food. How can they consume a toxic mushroom with no ill effects?

Timothy Johns (1990) gives insight as to how the people of Sanada Town may consume these poisonous mushrooms. Johns explains that humans have unique cultural traits that shape their interactions with chemical constituents from living organisms. One of these cultural traits is language, which provides a means of transmitting and retaining cultural knowledge. Knowledge of beni-tengu-take preparation and consumption is part of Sanada Town's oral tradition.

Simple technological innovations, such as cooking, also are cultural traits that have made humans less susceptible to the dangers of dietary toxins (Johns 1990). Long ago, people of Sanada Town discovered that boiling and pickling provide ways to increase the solubility of these mushroom toxins. By increasing the solubility of the toxins, they successfully remove the toxins and render the mushrooms edible.

There are other ways these people prepare *Amanita muscaria*. Some grill the caps while others dry the mushrooms then crumble the caps into food as a seasoning. These local practices of consuming *A. muscaria* have not been accurately documented nor has the effectiveness of these extraction processes been tested scientifically.

Here, I document traditional methods of *Amanita muscaria* collection, preparation, and consumption in the Nagano region of Japan. My objective was to determine the efficacy

of traditional practices in removing the isoxazole compounds responsible for beni-tengu-take's toxicity. Through the course of this study, I address the following questions:

Ethnomycological Questions:

1. Who eats *Amanita muscaria*?
2. How is *A. muscaria* prepared?
3. How much and how often are these mushrooms consumed?
4. Why is *A. muscaria* eaten?

Biochemical Questions:

1. How effective are traditional methods in detoxifying *A. muscaria*?
2. When are *A. muscaria* rendered edible?
3. Are all methods of detoxification equally effective?

1.1 THE STUDY MUSHROOM (*Amanita muscaria*)

Amanita is a well-known genus in Amanitaceae with 103 species (Singer 1975). Most *Amanita* species are mycorrhizal and, therefore, are found in wooded areas. *Amanita* ranges throughout the world, but attain their greatest diversity in warm, temperate zones (Arora 1986). Dioscorides coined the generic name ἀμανίγα (æm↔'naɪta) to describe a fungus found on Mount Amanon, Cilicia (Jenkins 1977). The specific epithet, muscaria, was first used by Linnaeus and refers to the musca fly (Simpson and Weiner 1989). Common vernacular names of *A. muscaria* include soma, fly agaric, and fly amanita. Japanese refer to *Amanita muscaria* as either beni-tengu-take (long-nosed, red-faced goblin mushroom), or hai-tori-take (fly-killer) (Imazeki 1973, Imazeki and Honda 1984, Imazeki and Wasson 1973, Ott 1976b).

A. TAXONOMY AND CLASSIFICATION

Since Elias Fries' fundamental work on fungal taxonomy, the amanita have been a well-defined group of Basidiomycetes. First included in the broadly-circumscribed *Agaricus*, then separated with the white-spored agarics into the tribe Leucosporae, *Amanita* has remained well circumscribed through the reordering of fungal taxonomy that followed the analysis of microscopic fungal characteristics (Beutler 1980).

Amanitaceae, as defined by Singer (1975), includes only two genera, *Amanita* and *Limacella*. Microscopic characteristics that define this family include the following:

a) Bilateral hymenophoral trama (tissue of the gills is organized so that the cross section shows hyphae diverging from the midline bilaterally, in the form of an inverted "V").

b) Spores white, thin-walled, round, and without ornamentation, mostly medium sized to large, and with a distinct hilum where the spore was attached to the basidium.

c) Hyphae are inamyloid (they do not react with Melzer's solution to give a dark color). Melzer's solution is a solution of iodine and KI in chloral hydrate that gives a blue-gray color with starch in the cell walls of hyphae or spores.

The breakthrough in *Amanita* taxonomy was Gilbert and Kuhner's (1928) discovery of the Melzer's reagent reaction correlation with the degree of cap margin striation. The formation of a blue-gray color in the spore wall with Melzer's was correlated with the absence of striation on the cap margin. Conversely, the presence of striation correlated with a negative (inamyloid) reaction of the spores. All *Amanita* species fell into one of two groups. These two characteristics form the basis for the division of *Amanita* into two subgenera, *Lepidella* for the former and *Amanita* for the latter. All treatments of the genus have maintained this subgeneric division. *Amanita muscaria* is a member of the subgenus *Amanita*.

Below this rank, the classification is less clear. Part of the problem is that subgeneric classifications have been made based on collections in a limited area and have failed to represent the true diversity within the genus. This makes any such subdivision of the

subgenera suspect. The distinctions in subgenus *Amanita* rest primarily on the presence of a basal bulb and a reduced volva in section *Amanita* contrasted with a very distinct saccate volva but lack of a bulb in *Vaginatae*. *Amanita muscaria* is a member of the section *Amanita*.

Jenkins (1977, 1986) found no valid type specimen for *Amanita muscaria*, one of the most widespread and well-known species in the genus. He designated a neotype based on the original description (Jenkins and Petersen 1976). Jenkins' monograph of section *Amanita* provides a good critical evaluation of the worth of characters (e.g. pileus color) for section *Amanita*. Bas' (1969) monograph does the same for the section *Lepidella*.

B. MORPHOLOGICAL DESCRIPTION

Amanita muscaria is a cosmopolitan basidiomycete found throughout the Northern Hemisphere in pine, spruce, fir, birch, and aspen forests and in pine plantations of Australia and New Zealand. The following morphological description is based on my field observations in Sanada Town, Japan.

Amanita muscaria (Figure 1.1) is a medium-sized to large terrestrial basidiomycete fungi whose fruiting body is found as solitary or scattered to densely clustered throughout birch forests and pine forests. *A. muscaria* pileus (caps) measure from 5 to 30 (sometimes 40) cm broad and are round becoming convex and finally flat or slightly depressed. The surface is viscid when wet and the color quite variable. In Japan, where this study takes place, the bright red, scarlet, to orange-red A. muscaria var. muscaria often fades to

yellow-orange with age and exposure to sunlight. A dense coating of universal veil covers the pileus of young basidiocarps. This veil tears with growth to form the characteristic white warts associated with many members of this genus. These universal veil remnants are usually white (except for *var. flavivolvata* that has yellow warts), flatten with age, and often wash off in the rain. The margin is usually somewhat striate. The flesh is firm when young but softens in age. All parts of the basidiocarp are white except for the bright red peelable cuticle and the tissue directly below the cuticle that is often a light yellow in color. The broad white gills are adnate to adnexed or free and are closely spaced. White spores are 9-13 x 7-9 microns, broadly elliptical, smooth, and are not amyloid (staining blue, gray, or black in iodine solution).

Amanita muscaria stipes (stalks) measure 5 to 20 (sometimes 30) cm long and are 1 to 4 cm thick at the apex, tapering upward or equal with a basal bulb up to 6 cm broad. The stipe is white and smooth but somewhat scaly below the annulus. Stipe tissue is tough and fibrous without and hollow or filled with softer central mycelial pith within. A membranous partial veil forms a thin, persistent median to superior skirt-like annulus or ring on the stalk that may collapse or tear off with age. The annulus is white and its margin is often torn or toothed. Remnants of the universal veil form a scaly volva at the apex of the bulb. The volva characteristically has 2 to 4 (sometimes more) concentric rings.

Figure 1.1 *Amanita muscaria* photographed in Sanada, Japan

C. FIELD IDENTIFICATION AND COLLECTION

This brilliant red mushroom needs no introduction. Its caricature appears in children's books, calendars, calling cards, posters, incense holders, candles, and key chains. The bright red color with white warts is its outstanding field characteristics. The white gills, presence of an annulus, and scaly volva separate it from other bright red mushrooms (notably the Russulas). I used Jenkins's (1977) taxonomic and nomenclature study of the genus *Amanita* section Amanita for North America, Imazeki et al's (1988) Fungi of Japan, and David Arora's (1986) Mushrooms Demystified to aid in my identification of *Amanita muscaria*. To confirm my determinations, Dr. Ohmasa at the Shinshu University Department of Mycology determined my collection material to be *A. muscaria*.

D. ETHNOMYCOLOGICAL USE OF *Amanita muscaria*

Use of *Amanita muscaria* for ritual, hallucinogenic, or other purposes is found in several cultures throughout the world. Many of these cultures are far removed from one another, but these far-flung practices may be related.

1. **Siberia** – Several ethnomycological studies verified reports of Siberian tribes eating *Amanita muscaria* as an intoxicant or hallucinogen. For centuries, the Khanty, Koryak, Chukchi, Eskimos, and Russians of the Kamchatka peninsula consumed *A. muscaria* (Saar 1991a). Saar (1991a,b), who documented 15 ways of consuming *A. muscaria,* noted that people ingested small dried buttons as a stimulant but only shamans were allowed to consume larger, hallucinogenic-inducing mushrooms. Early travelers to West

Siberia commented that when *A. muscaria* is eaten, the hallucinogenic principles are excreted through the urine, and for the poor individual who had no mushrooms, drinking the urine of someone who had eaten such mushrooms achieved similar results. Saar (1991a) also observed that reindeer are extremely fond of *A. muscaria* and human urine and become intoxicated when they consume either substance.

2. **Europe** – *Amanita muscaria* was used in homeopathic and herbal practice in early 19th century European and American medicine. An extract of fly agaric was prescribed for night sweats due to debilitating disease. The dose given was 5 drops of a 1% solution of extract (Felter and Lloyd 1898). Culbreth (1927) reported that this mushroom reduces force and frequency of pulse, contracts muscles of intestines and bladder, increases abdominal secretions, causes labored breathing, paralysis, and death. He also said *A. muscaria* is given for intestinal torpor, duodenal catarrh, diabetes, and as an antidote to atropine to replace physostigmine. Homeopaths currently use *A. muscaria* to treat chorea and skin afflictions. It is specific for bunions, sunstroke, and reportedly clears up some types of cataracts (Ramsbottom 1953, Ying et al. 1987)

3. **Indus Valley** – About 1500 B.C., a people called the Aryans came from the north through Afghanistan and occupied the historic Indus River Valle. The bulk of our knowledge of the Aryans is derived from a collection of over 1000 hymns (known as the Rig Veda) composed by their priests and sung during worship. Wasson (1968, 1970, 1971) relying on the assistance of the Vedic scholar O'Flaherty (1969) has presented very persuasive arguments that the Soma of the Rig Veda was in reality the Fly Agaric,

Amanita muscaria. The Sanskrit scholar Ingalls (1971a,b) agreed with Wasson's conclusions. Nonetheless, identification of Soma has been hotly debated (Brough 1971, La Barre 1970, Riedlinger 1993).

4. **Northwest Pacific (Canada and United States)** – Weil (1977) and Ott (1976a,b) describe the recreational use of *Amanita muscaria* and *A. pantherina* among the North American drug subculture. Weil claims this use of *Amanita* dates back only to the attempts to identify the ancient Aryan intoxicant Soma with the fly agaric (Wasson 1968) and to numerous popular accounts of the psychoactivity of this species (Chilton 1975, Furst 1972, Guzman et al 1976, Lincoff and Mitchel 1977, McDonald 1978, Ott 1976a, Tyler 1979); however, Wasson (1979) reports evidence of the Ojibway people and possibly others using *A. muscaria* for divine guidance in their shamanic rituals.

Ott (1976b) notes a culinary use of *Amanita pantherina* in the state of Washington. Users collect a large quantity of *A. pantherina*, peel the skins from the pilei of the mushrooms and discard them. The mushrooms subsequently are parboiled, the water is discarded, and the mushrooms are canned for future use. Apparently, users in Washington are unaware of the toxicity of fresh specimens (Ott 1976b).

5. **Mexico and Central America** – Lowy (1972, 1974) reports evidence of *Amanita muscaria* symbolism in Mayan codices and various legends in Guatemala and Mexico.

6. **Japan** – There are several ethnomycological references to a Japanese pickling process for *A. muscaria*. All of these references can be traced to Wasson (1968, 1973). Tsunoda et al. (1993a) investigated the change in ibotenic acid and muscimol contents in *Amanita muscaria* during drying, storing, or cooking. They gave no account of their ethnographic methodology nor did they report the effects of grilling *A. muscaria* caps. Tsunoda et al (1993a) reported their findings in Japanese and used an obscure HPLC method that required an extremely low pH.

E. IBOTENIC ACID AND MUSCIMOL

Ibotenic acid and muscimol (Figure 1.2) are responsible for the toxicity of *Amanita muscaria*. Swiss, British, and Japanese scientists independently isolated these two isoxazole compounds in the early 1960s (Bowden and Drysdale 1965; Eugster 1967, 1969; Good et al. 1965; Muller and Eugster 1965; Takahashi and Onda 1985; Takemoto 1967; Takemoto and Nakajima 1964; Takemoto et al. 1964a,b).

Ibotenic acid and muscimol are conformationally restricted derivatives of glutamic acid and GABA (gama-aminobutyric acid) respectively (Figure 1.2). Brehms et al. (1972, 1997, 1998) compared the x-ray crystallography of muscimol to GABA. Borthwick and Steward (1976) compared the CNDO-calculated (complete neglect of differential overlap) conformations of ibotenic acid to solution conformations of glutamic acid. GABA is related to glutamic acid in the same way that muscimol is related to ibotenic acid, by decarboxylation (Baker et al. 1971, Chilton 1994).

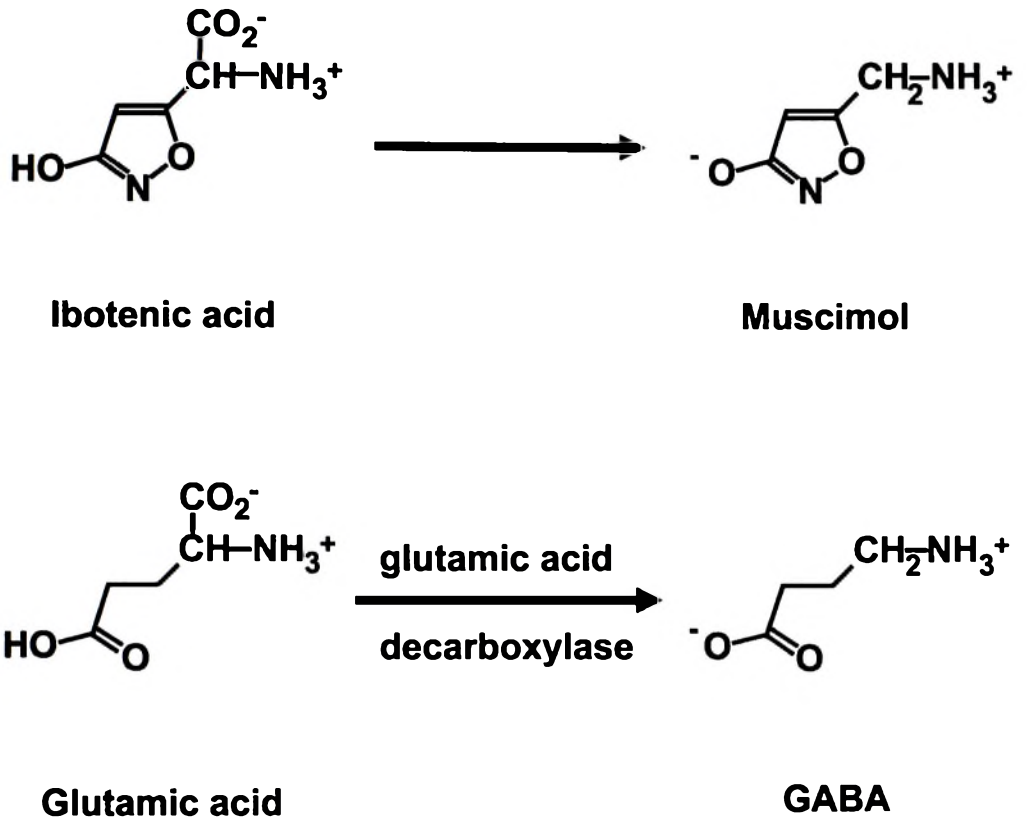

Figure 1.2 Decarboxylation of ibotenic acid and glutamic acid.

1. SYMPTOMS AND PHARMACOLOGY

Ibotenic acid and muscimol evoke hallucinations, delirium, muscular spasm, and sleep (DeFeudis 1980, Theobald et al. 1968, Waser 1967). Experiments in animals and humans support a central nervous system (CNS) role for muscimol and ibotenic acid as GABA receptor agonists (Janis 1991). Unlike GABA, the natural neurotransmitter it mimics, muscimol exerts pronounced CNS effects (Biggio et al. 1977a,b). The threshold for observation of CNS disturbances in humans is about 6mg/kg muscimol or 5 to 10 times that amount of ibotenic acid (Theobald et al. 1968). This dose is potentially available in a single *Amanita muscaria* or *A. pantherina* cap. The ready decarboxylation of ibotenic acid complicates interpretation since the ibotenic/muscimol ratio may change during experiments. A major portion of the activity of administered ibotenic acid is due to muscimol generated *in situ* (Chilton 1994). The acute LD_{50} of muscimol in rats ranges from 4.5mg/kg i.v. to 45mg/kg by mouth. Experiments in dogs suggest that the effects of ingestion of 20 mg/kg/day orally are not cumulative (Theobald et al. 1968). The effect of ibotenic acid on the glutamate-mediated peripheral nervous system of snails (Walker et al. 1971) and insects (Lea and Usherwood 1973) and behavioral effects of muscimol on the possum have been studied (Camazine 1983).

In humans, effects are measurable about 1 hr after ingestion of 7.5 to 10mg of muscimol or 50 to 90mg ibotenic acid (Chilton 1975, 1994; Leonhardt 1949). These effects continue 3 to 4 hrs with some residual effects for up to 10 hrs later (Chilton 1975, Theobald et al. 1968). Subjects slow in performing tasks and appear tired, muscle spasms in the extremities occur, and stomach upset and vomiting is common. There is no

significant change in pulse rate or blood pressure. Subjective changes of state are frequent, but, while some individuals relax, others become tense. Viewing oneself from outside one's own body and a sense of levitation are common experiences in both voluntary and accidental intoxications (Rumack 1994). Deaths from *A. muscaria* or *A. pantherina* are rare. Ingestion of 20 large *A. muscaria* has been survived (Mitchel 1992); however, it requires consuming 10 or more of these mushrooms to produce the rare fatalities (Lincoff and Mitchel 1977).

Muscimol lacks cholinergic effects at the neuromuscular junction (Chilton 1994), and low doses of muscimol affects the EEG of cats and rabbits (Scotti de Carolis et al. 1969, Theobald et al. 1968). These observations support a localization of muscimol action in the CNS rather than in the peripheral nervous system. Muscimol and ibotenic acid administered to rats interperitoneally affect brain levels of serotonin, noradrenalin, and dopamine (Koenig-Bersin et al. 1970, Waser 1971).

Muscimol, like GABA, the neurotransmitter it mimics (Figure 1.3), is a powerful inhibitor of firing of central neurons (Johnston et al. 1968, Johnston 1971). Ibotenic acid, the precursor to muscimol, is an active neurotransmitter agonist in its own right. It mimics the excitatory amino acid glutamic acid. Unlike the true neurotransmitters, muscimol readily passes the blood-brain barrier (Moroni et al. 1980). Neither muscimol nor ibotenic acid is removed from the environment of the receptor by the GABA or glutamate active uptake system (Chilton 1994). Inefficient removal of these GABA-mimics once they have passed the blood-brain barrier may be an important contributing

factor to their CNS effects (Balcar and Johnston 1972, Krogsgaard-Larsen and Johnston 1975). Enzymatic decarboxylation of glutamic acid into GABA unifies the true neurotransmitter pair. The glutamic acid mimic, ibotenic acid, similarly undergoes decarboxylation to give the GABA mimic, muscimol. Ibotenic acid decarboxylation is spontaneous, and, unlike glutamic acid decarboxylation, does not require an enzyme (Chilton 1994). However, the decarboxylation of ibotenic acid is accelerated in the mouse brain by a pyridoxal phosphate-dependent enzyme (Nielsen et al. 1985). It is probable that ibotenic acid is a substrate for brain (S)-glutamic acid decarboxylase (GAD).

GABA binding to its receptors is involved in the regulation of a number of physiologic functions, including cardiovascular mechanisms and sensations of pain and anxiety. Loss of GABA receptors is involved in epilepsy, Huntington's chorea, and spasticity (Chilton 1994, Falch et al. 1984). Muscimol potentially could treat these disorders (Krogsgaard-Larsen 1984). Ibotenic acid and muscimol have become important tools in the study of the gabaminergic nervous system (Krogsgaard-Larsen et al. 1988, 1986, Krogsgaard-Larsen 1992) and have been used in over 1,000 neuropharmacologic studies since their discovery in 1965.

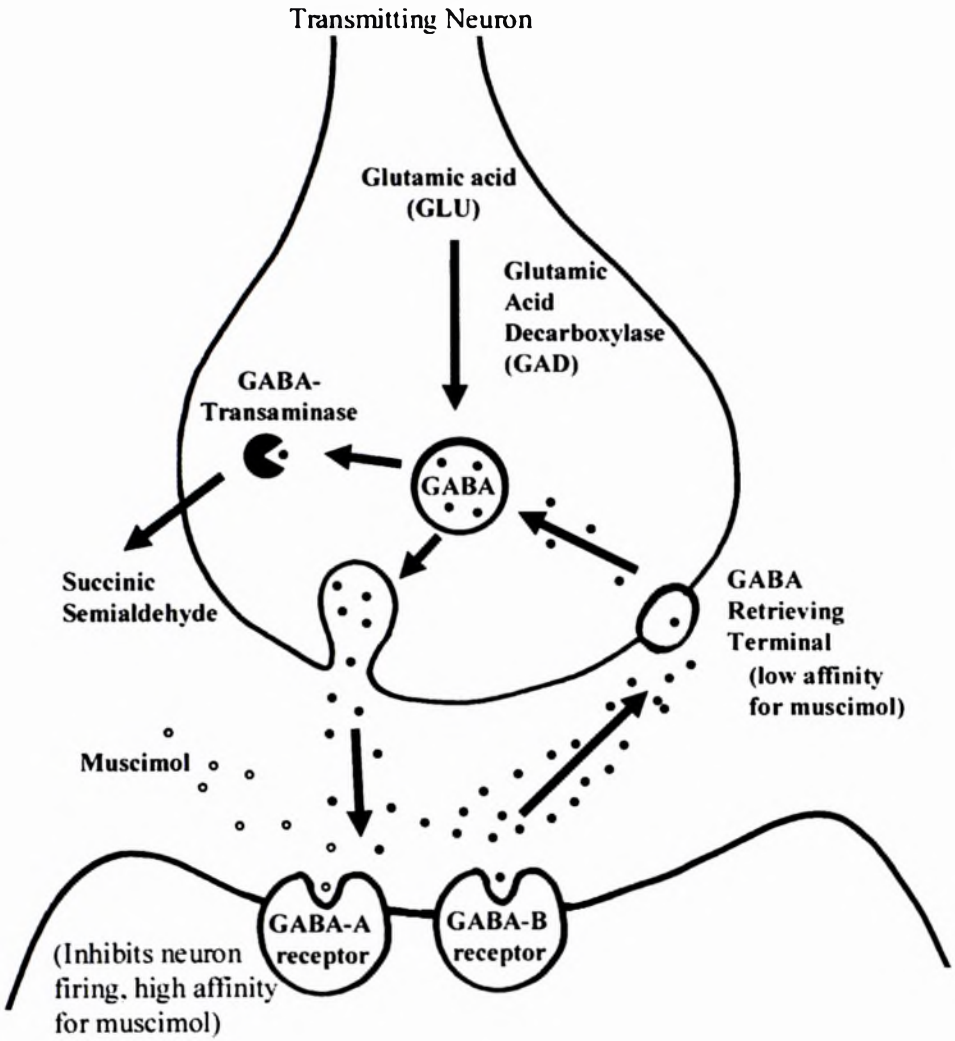

Figure 1.3 Effects of muscimol on GABA metabolism in the synaptic cleft

2. METABOLISM OF IBOTENIC ACID AND MUSCIMOL

Muscimol absorbed into the blood stream is excreted in the urine within six hrs (Ott et al. 1975). A substantial fraction of ingested ibotenic acid is excreted unmetabolized. Virtually no muscimol is excreted when pure ibotenic acid is ingested, but muscimol is detected in the urine after eating *Amanita muscaria*. The ibotenic acid that does pass through the body is excreted rapidly between 20 and 90 minutes after ingestion (Chilton 1975). Major symptoms occur only after 30 to 60 minutes and reach their greatest intensity after excretion of the majority of the consumed ibotenic acid. Strong intoxication lasts more than three hours after the peak in excretion of ibotenic acid (Chilton 1994, Hall and Hall 1994, Rumack 1994).

3. DISTRIBUTION OF IBOTENIC ACID AND MUSCIMOL

Examinations for the presence of ibotenic acid and muscimol have been performed on 3 European and Japanese *Amanita* spp. and on 33 North American species (Benedict et al. 1966, Chilton and Ott 1976, Beutler and Der Marderosian 1981). These two compounds appear to be characteristic of the Section Muscaria of the genus *Amanita* only.

Quantitative data on concentrations of ibotenic acid and muscimol present in these mushrooms are not readily comparable. Some assays used mushroom fresh weight, some dry weight, and some used the total of ibotenic acid plus muscimol. Estimates based on isolation and weighing of ibotenic acid tend to be low because of decarboxylation of ibotenic acid during isolation. Eugster et al. (1965) isolated 300 ppm ibotenic acid from Swiss *A. muscaria* and estimated true content of ibotenic acid to be near 500 ppm with no

muscimol present in fresh mushrooms. Bowden and Drysdale (1965) quantified 50 ppm muscimol from fresh European *A. muscaria*. Takemoto et al. (1964b) isolated 25 ppm ibotenic acid from *A. muscaria* and 210 ppm from *A. pantherina*. Chilton and Ott (1976) isolated 50 ppm ibotenic acid from fresh North American *A. pantherina*. Repke et al. (1978) analyzed dried *A. pantherina* and found 0.046% muscimol and 0.002% ibotenic acid. These results show the extensive decarboxylation and potentiation of toxicity that may accompany drying or storage since muscimol is five to ten times as active as ibotenic acid. Estimates by a chromatographic spot intensity method found a total of 0.18% ibotenic acid plus muscimol in dried *A. muscaria* and 0.46% of the two toxins in dried *A. pantherina* (Benedict et al. 1966). Yamaura and Chang (1988) found 660 ppm ibotenic acid and 280 ppm muscimol in fresh *A. pantherina*. The slow loss of both ibotenic acid and muscimol on storage may explain the differences in assays (Tsunoda et al. 1993a). Neither was detectable in samples dried and stored for 7 years. Isolation yields suggest that European *A. muscaria* is potentially more toxic than any North American species. However, since muscimol is five to ten times more toxic than ibotenic acid, actual toxicity depends on the extent to which ibotenic acid has been decarboxylated into muscimol during preservation, preparation, and storage.

Tsunoda et al. (1993b) reported changes in the concentration of ibotenic acid and muscimol in the fruiting body of *Amanita muscaria* during the reproductive stages.

The red and yellow pigments of *A. muscaria* are localized in the peelable skin of the cap (Chilton 1994). Six of the pigments have been isolated and identified as betalamic acid

conjugates of amino acids (Dopp and Musso 1974, Talbot and Vining 1963, Mueller et al. 1996, Mueller 1997). One of the yellow pigments is the conjugate of ibotenic acid (Dopp et al. 1982). Assays of ibotenic acid have not taken this source of ibotenic acid into account. The yellow region just under the red cuticle of fresh *A. muscaria* contains more free ibotenic acid than white portions of the mushroom (Gore and Jordan 1982).

1.2 PHYSICAL GEOGRAPHY

A) LOCATION

I conducted fieldwork in October 1999 within the town of Sanada. Sanada is located 5km north of Ueda City in Nagano Prefecture in the middle of Honshu Island (Figure 1.4). Sanada has an area of approximately 18,200 ha and is the third largest town in the east region of Nagano Prefecture (Sanada-machi 1998). I collected *Amanita muscaria* in the public forested land of Mt. Nekodake in the Sugadaira alpine prairie district of Sanada (36° 32'N, 138° 22'E, 1500m), and conducted most interviews near Sanada town hall (36° 26'N, 138°18'E, 700m).

B) GEOLOGY AND SOILS

Sanada lies in the high mountains of the Fossa Magna fracture belt. The topography within town limits varies from the steep, deeply dissected slopes of the 2300m mountain range to the 60km^2 alpine prairie of the Sugadaira suburb at 1200m elevation and the fertile valley of the downtown area at 700m elevation. Soils in Sanada include well-drained and poorly drained brown ultisols with alluvial deposits (entisols) along rivers.

C) CLIMATE

Sanada is located within the alpine mountains that protect the Pacific coast from harsh winter weather. Consequently, Sanada receives abundant snowfall each winter. The mean annual temperature is 9°C. Since Sanada is located inland, the mean annual rainfall

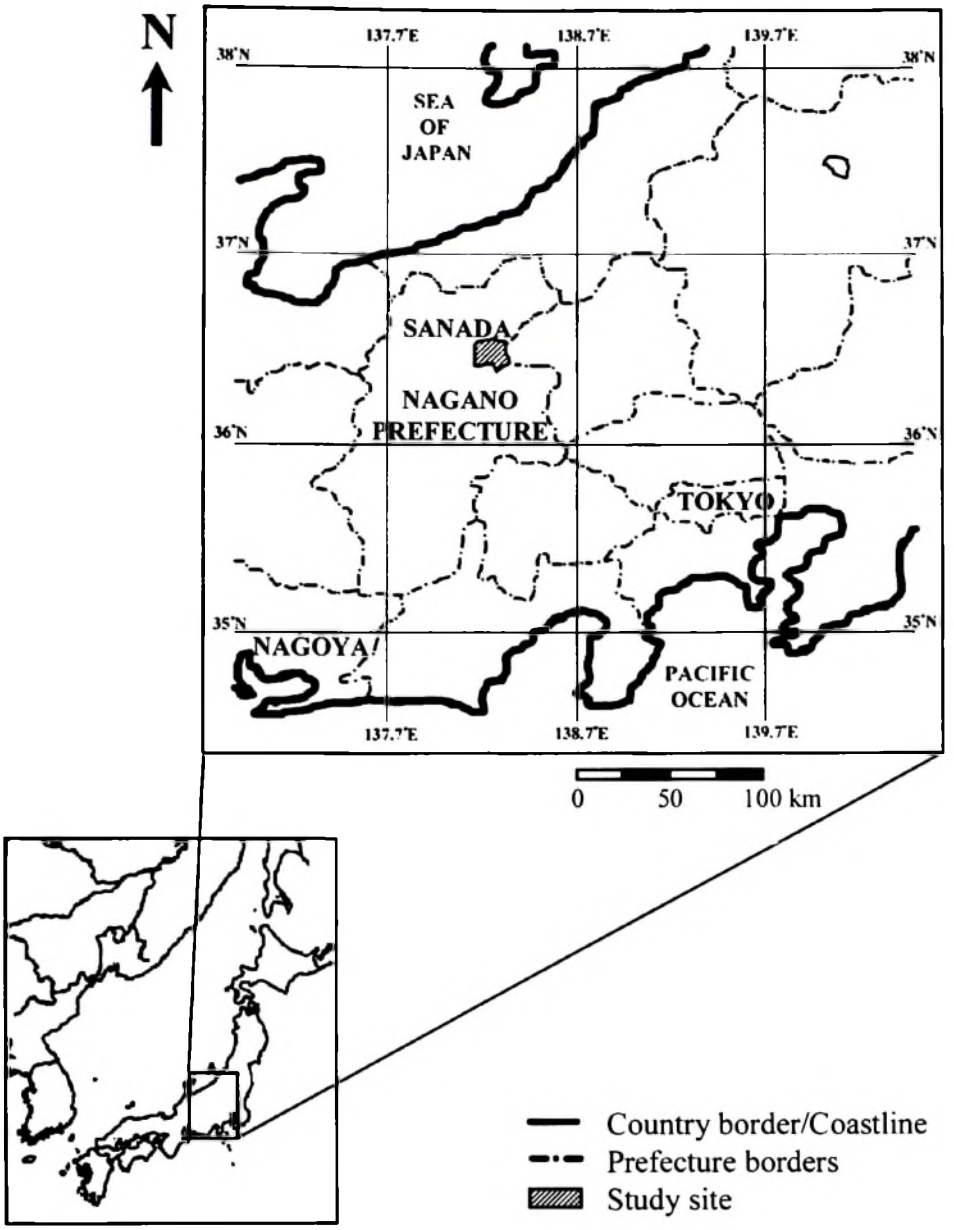

Figure 1.4 Map of Japan with location of Sanada Town study area

is only 800mm; however, the Sugadaira alpine prairie receives abundant snowfall. The mean annual snowfall for Sugadaira is $87m^2$ (Sanada-machi 1998). See Figure 1.5 for a summary of climate conditions.

D) VEGETATION

Sanada contains cool-temperate broad-leaved deciduous (summer-green) forest with some boreal forest found at higher elevations. Forests cover sixty three percent of the land within the boundaries of Sanada (Sanada-machi 1998). *Amanita muscaria* grows exclusively in birch tree forests above 1400m. Large stands of *Betula platyphylla* Sukaczev var. *japonica* (Miq.) Hara dominate this forest type. *Rhododendron* sp. and *Sasa veitchii* (Carrie) Rehder typify the shrub layer and ground cover for this forest type. This forest type covers 2% of the land within Sanada Town (Sanada-machi 1998).

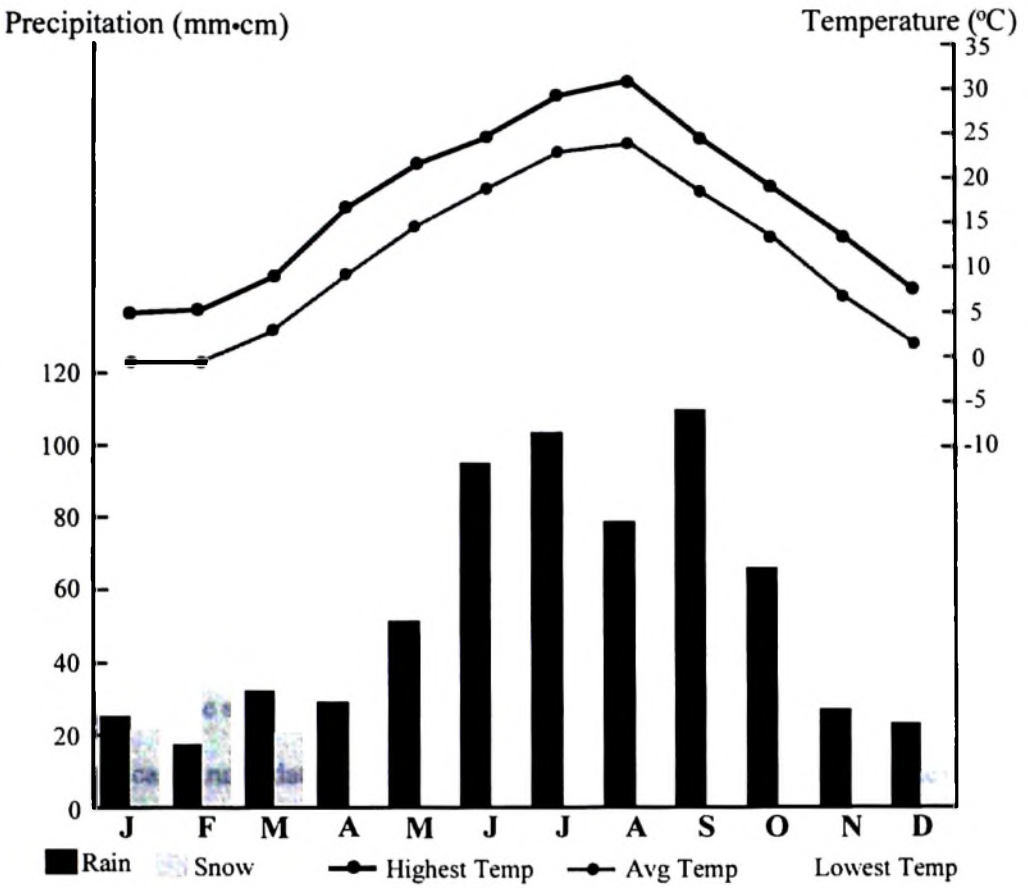

Figure 1.5 Climate diagram for Sanada Town, Japan (after Sanada-machi 1998)

1.3 HUMAN GEOGRAPHY

THE PEOPLE

I worked with rural Japanese people of Sanada Town in the mountainous Nagano Prefecture. Sanada town has a population of 11,789 people (Sanada-machi 1999). This population geographically is separated into two close-knit communities; the smaller alpine-plane ski district of Sugadaira and the larger town center of Sanada located in the valley below. I conducted interviews within both of these communities.

LOCAL HISTORY

Sanada Town is the result of a 1958 merger of four villages, Sanada village, Osa village, Motohara village, and Sochi village. Sanada Town is famous as the birthplace of the Sanada Clan who ruled this area and were active during the Warring States Period (around the 16th century). The Sanada Clan were high-ranking samurai military commanders whose strategies and bravery are legendary throughout the country. Old temples and castle ruins dating back to this period remain standing throughout the town. Tragic stories and legends of the Sanada samurai are still told throughout the generations [Sanada-machi 1998].

LAND USE

Forests cover 63% of Sanada Town. Less than 8% of the land is used as farmland, 8% as pasture, and 2% of the land is developed for housing. Rice, lettuce, cabbage, apples, peaches, potatoes, soybean, wheat, red beans, honey, silk, cultivated flowers, and cultivated mushrooms compose major crops of Sanada Town. Rice and vegetable

production are centered along rivers while fruit trees are planted on mountain slopes [Sanada-machi 1998].

ECONOMY

The small town of Sanada is experiencing an increase in industrialization and subsequent decrease in farming. Less than 22% of Sanada Town's population relied on agriculture for income in 1995, a decrease from 67% in 1960. Sanada Town now relies heaviest on Industry (US$110 million per year) and Business (US$85 million per year). Tourism adds US$53 million per year to the income of Sanada Town with agriculture averaging US$33 million per year. 70% of farmers in Sanada Town are small-scale farmers. Mushroom cultivation accounts for US$5.1 million per year in Sanada Town [Sanada-machi 1998].

CHAPTER II. TRADITIONAL JAPANESE COLLECTION, PREPARATION, AND CONSUMPTION OF BENI-TENGU-TAKE (*Amanita muscaria* (L. ex Fr.) Pers. ex Hook.)

2.1 INTRODUCTION

Amanita muscaria (L. ex Fr.) Pers. ex Hook. (Amanitaceae) is a common poisonous mushroom in many parts of the world. Several cultures employ *A. muscaria* as a hallucinogen but most people consider it a mere poison (Lowy 1972, 1974; Saar 1991a, b; Wasson 1968). Residents of Sanada Town, Japan employ three primary methods of detoxifying *A. muscaria*: pickling, grilling, and drying. A fourth and seldom-encountered method involves making a tincture by soaking the mushrooms in alcohol. Each method of preparation is not equally effective in removing the hallucinogens and an individual's reasons for consuming mushrooms prepared in different ways may vary.

Here, I discuss current uses of *Amanita muscaria* in Sanada Town, Japan as a food. Wasson (1973) briefly mentioned the pickling and grilling processes, but this study more thoroughly documents methods of *A. muscaria* collection, preparation, and consumption in Sanada Town. In addition, I address the question of why these people eat a poisonous mushroom.

2.2 METHODS

A. ETHNOMYCOLOGICAL DATA COLLECTION

In June of 1998, I traveled to Japan to initiate this study. During this month-long visit, I searched Japanese literature at Nagoya University, Nanzan University, and Shinshu University's School of Agriculture libraries. I chose Sanada Town as the primary study site because of its close proximity to Ueda City. Wasson (1973) mentioned Ueda's *Amanita muscaria* pickling practice. I found many people in the neighboring Sanada Town who consumed *Amanita muscaria* and who were willing to share with me their uses for this mushroom.

During this initial trip, I met town officials and performed the formal Japanese greetings. I explained my research objectives as well as the potential benefits my findings might have for their community. My knowledge of formal Japanese greetings and ability to speak Japanese helped establish professional relationship with the town officials and good rapport among community members. After approval from the town officials, I conducted preliminary interviews in Sanada-machi with ten informants.

I returned to Sanada Town for two weeks in October of 1999 to interview informants and to collect bulk samples of *Amanita muscaria* for chemical analyses. I conducted semi-structured interviews in Japanese with 123 informants, asking specific questions, but giving each participant freedom to interject and expound upon any given question or idea. Informants were not paid but were given small homemade *Amanita muscaria* trinkets I

made from polymer clay. During semi-structured interviews, I recorded uses of *Amanita muscaria* and inquired as to each method of preparation for human consumption.

I conducted several interviews in the field when I met *Amanita muscaria* collectors (Figure 2.1). Most of the 123 interviews occurred at three local mushroom exhibitions. The first day of each exhibition consists of a community-wide mushroom foray in the morning followed by an afternoon of mushroom sorting. Town officials invite local mushroom experts to help in the identification of mushroom species. On the following day, those mushrooms collected and identified are displayed in a public area. The local branch of the National Health Insurance sponsors these mushroom festivals to inform the people of edible and poisonous mushroom species with the hopes of reducing the number of annual mushroom poisonings. A mushroom feast follows the exhibition where all edible species of mushrooms are showcased in a variety of delicious dishes.

In all mushroom exhibitions, local experts labeled *Amanita muscaria* as a poisonous species. I watched people's reactions to this designation and listened to their conversations as they discussed whether they considered *A. muscaria* a truly poisonous species. I would then ask about the preparatory methods necessary to ensure the edibility of *A. muscaria*. Figure 2.2 shows the age and gender distribution for my informants interviewed in this manner.

I performed interviews with 10 especially knowledgeable informants. Interviews with key informants lasted approximately an hour during which I asked informants to

demonstrate their personal methods of *Amanita muscaria* preparation. I used a video recorder and camera to document interviews. A town official transcribed these interviews to give a more accurate account of what was said by informants.

B. MUSHROOM COLLECTING METHODS

My informants and I collected two bulk samples of *Amanita muscaria* during my second trip to Japan in October of 1999. My Japanese collaborators identified optimal collecting sites and showed how to collect in traditional manners. Ethnological vouchers were collected and deposited at Shinshu University and at FIU.

Figure 2.1 The author conducting a typical interview with a Japanese couple found collecting *Amanita muscaria*. A town official (author's immediate right) is transcribing the interview.

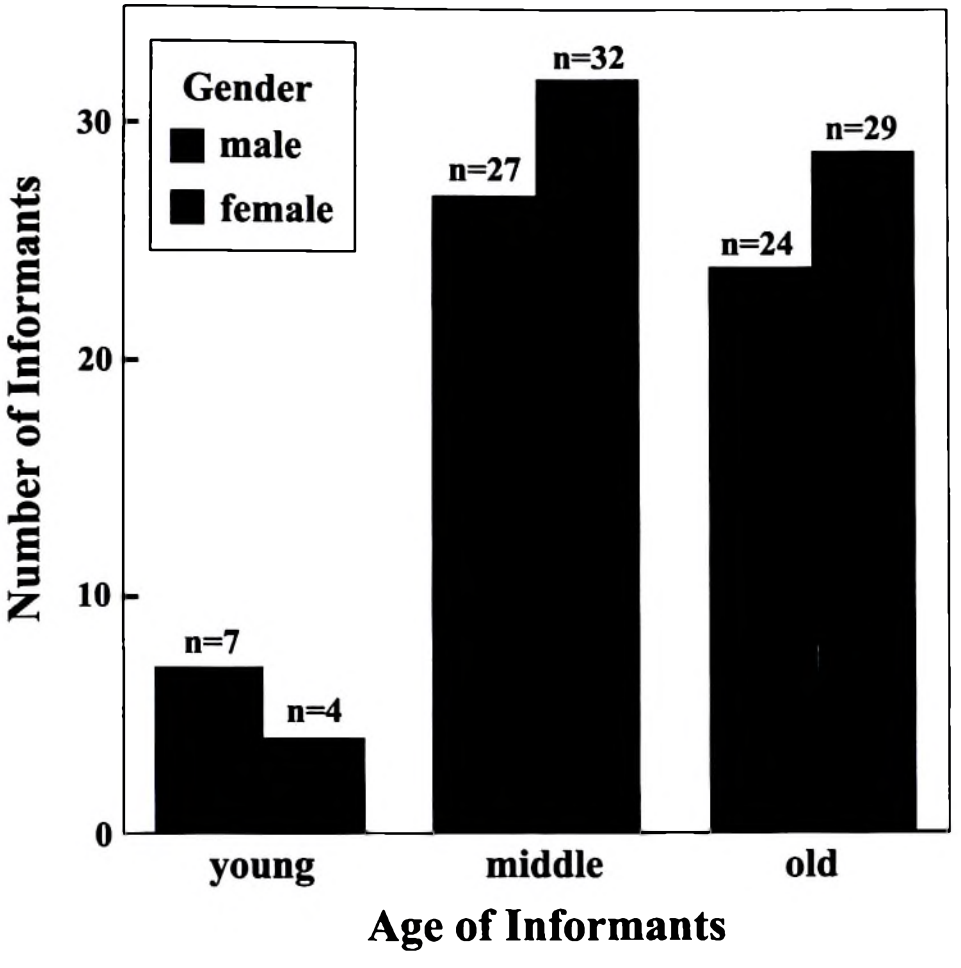

Figure 2.2 Age and gender of informants interviewed in this study

2.3 ETHNOMYCOLOGICAL RESULTS

A. VERNACULAR NAMES

The Japanese had two names for *Amanita muscaria*: beni-tengu-take and hai-tori-take. Beni-tengu referred to a popular red-faced, long-nosed goblin in Japanese demonology (Figure 2.3). This goblin had the ability to fly and is notoriously mischievous, both attributes of consuming too many of these mushrooms. Hai-tori literally translated to mean "fly-killer". The Japanese realized *A. muscaria's* fly-killing potential and used an infusion of mushroom in milk in the kitchen as an insecticide. Take means "mushroom" or "fungus".

Figure 2.3 Tengu goblin of Japanese demonology (from Nishimoto 1996)

B. GATHERING

Both men and women in Sanada Town gathered *Amanita muscaria* throughout the fall (Figure 2.4); however, most people enjoyed gathering early in the fruiting season. The local people knew *A. muscaria* grows under birch trees from late summer until snowfall and gathered mostly in the birch forests of the Sugadaira ski district between 1400 and 2000 meters. They did not discriminate between size and developmental stage when gathering these mushrooms. Some people preferred immature fruiting bodies (Figure 2.4b).

Figure 2.4 a) Collecting *Amanita muscaria* to use in the pickling process, b) Mr. Satou collects immature *A. muscaria* that appear completely white in color.

C. PREPARATION AND CONSUMPTION

1. DRYING

Collectors sometimes dried *Amanita muscaria* for later use. Informants placed whole mushrooms on large woven bamboo trays in direct sunlight for 2 to 3 days. Once dried, the mushrooms were inspected for mold or other contaminants. When sufficiently desiccated, these mushrooms were stored in bags within the house. A few informants crushed and sprinkled dried mushrooms into rice to enhance its flavor (Figure 2.5, Figure 2.6). Most people re-hydrated the dried mushrooms and pickled them as they would the fresh mushrooms. Dried mushrooms last several years.

2. PICKLING

While there were several variations to this pickling process, all households followed these four basic steps: boiling, washing, salting, and soaking. First, informants boiled the mushrooms for an average of 10 minutes (Figure 2.7a). Some informants performed up to three successive 5-minute boilings, each time throwing away the hot water. Many informants boiled mushrooms until they blanched white, but some left a little color in the mushrooms for aesthetic value. After boiling, all informants washed the mushrooms in running water for 1 to 3 minutes (Figure 2.7b). Informants then packed the mushrooms in salt (Figure 2.7c) and applied pressure (Figure 2.7d). Some weighed the mushrooms and used an equal amount of salt when performing this step. The informants mixed the mushrooms in the salt and packed this mixture into a glass container. Informants said it is important to leave the mushrooms in the salt for one month before consuming the pickles.

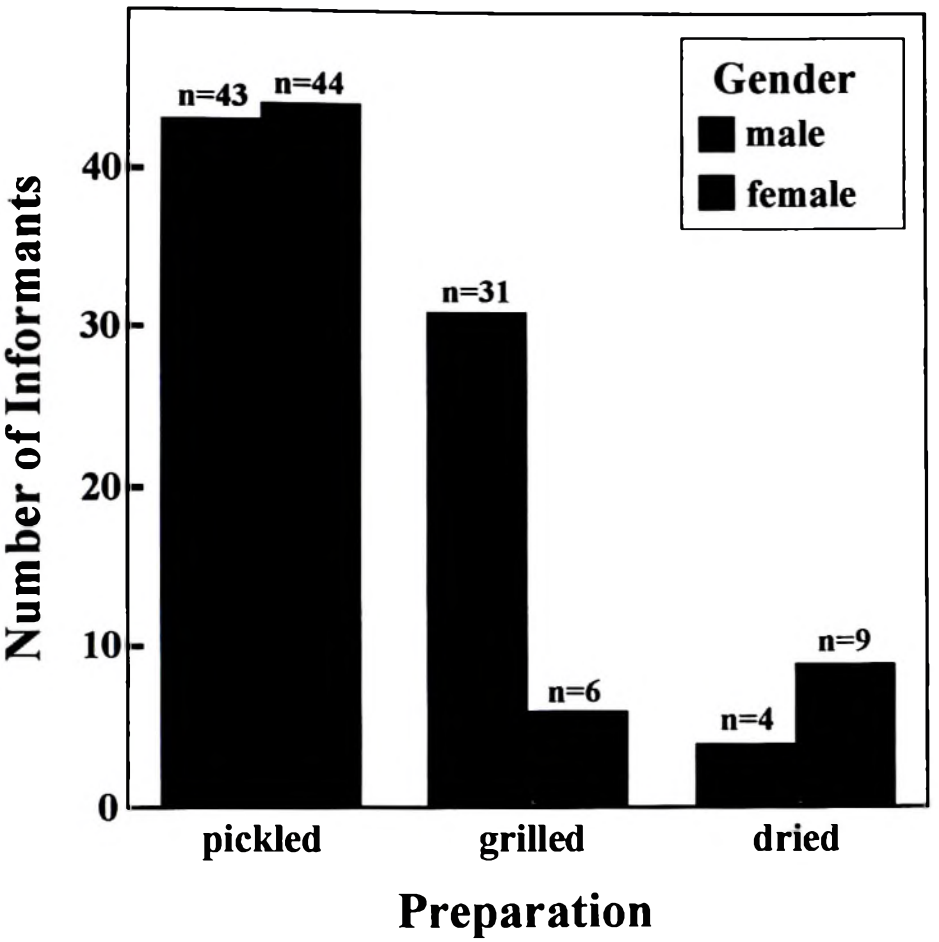

Figure 2.5 Gender and preferred method of preparation.

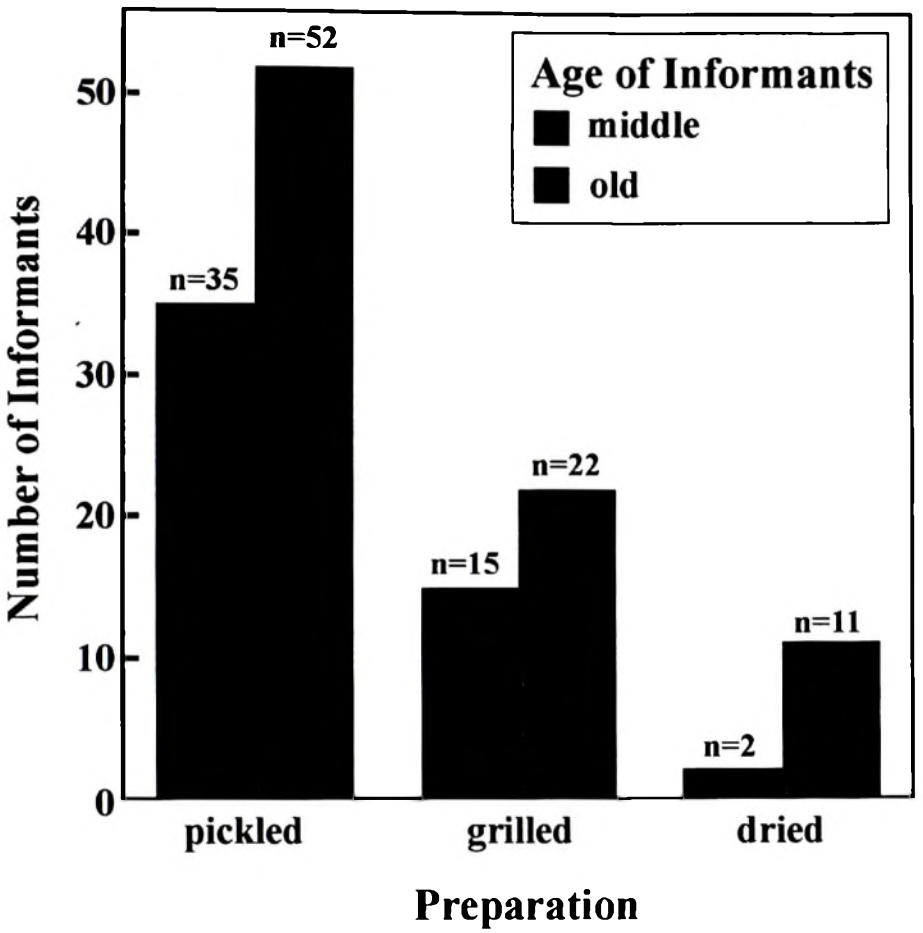

Figure 2.6 Age and preferred method of preparation.

Before consumption, informants removed the pickles from the glass container and soaked them in water for several hours to overnight to remove the salt. Then, informants ate these mushrooms as hors-d'oeuvre or added them to a variety of stir-fry, sauté, and rice dishes. Many families eat *A. muscaria* pickles as part of their New Year's celebration. Some restaurants, hotels, and inns in Sanada Town had these pickles on hand and would serve them to special guests. Many informants remembered seeing vendors selling these mushrooms in the early 20th century. Apparently, these pickles were quite a delicacy in those days and were served at festivals and special events.

3. GRILLING

Men of Sanada Town grilled *Amanita muscaria* caps (Figure 2.8) and usually discarded the fibrous stems. A few informants, however, grilled the stems because they are less toxic. Most knew of beni-tengu-take's toxicity and claim that they consumed grilled *A. muscaria* for its flavor and not its hallucinogenic effect. The people I observed consuming *A. muscaria* caps only ate a small quantity (up to 15 grams).

4. TINCTURE

Only one informant said he prepared *Amanita muscaria* by poking several small mushrooms into a bottle of *sake*, Japanese rice wine, and allowing them to soak for several days to a week before drinking the tincture. This informant said he would drink a small amount of this narcotic beverage for its relaxing effects.

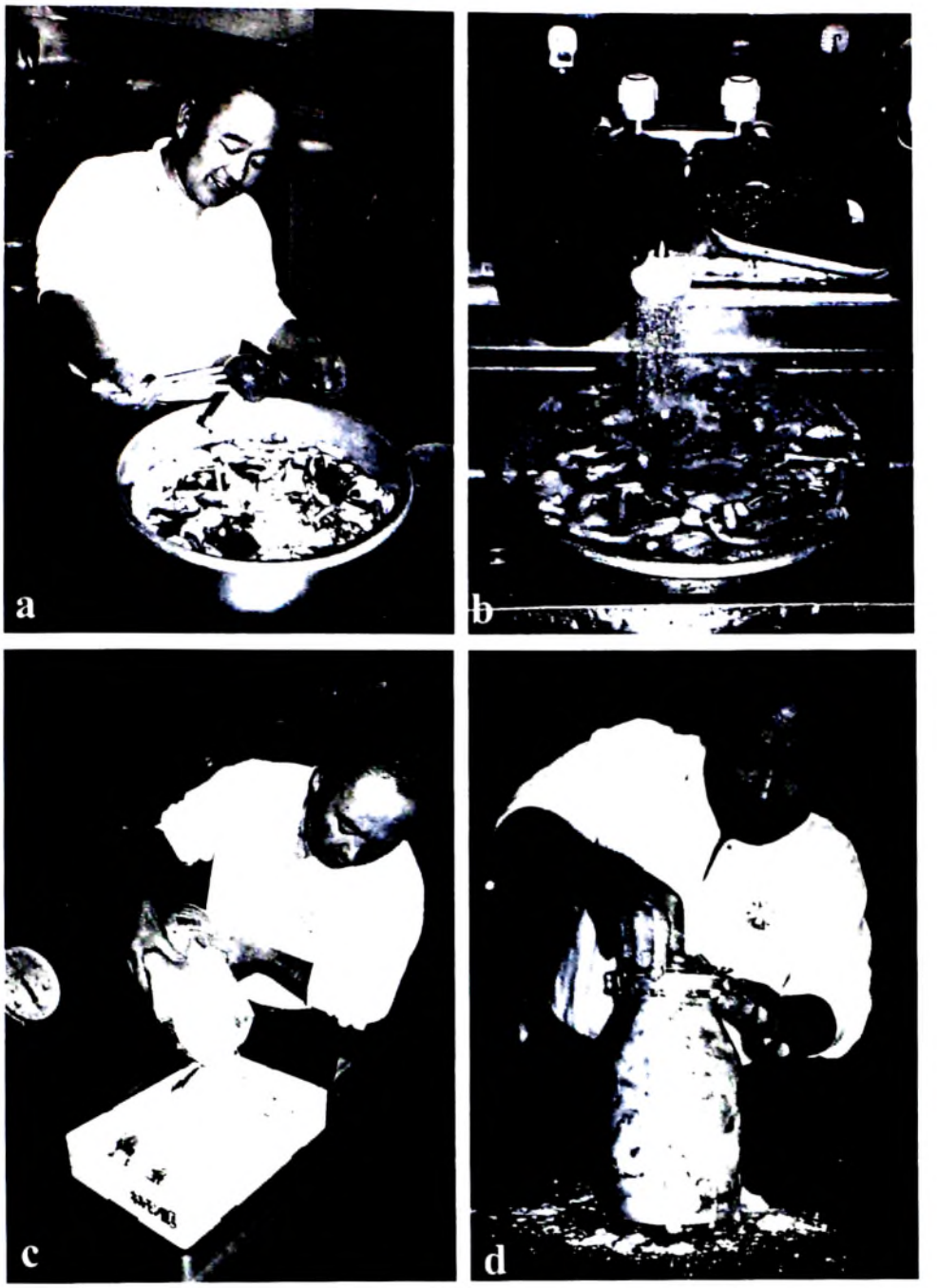

Figure 2.7 *Amanita muscaria* pickling process, mushrooms are a) boiled an average of 10 minutes, b) then washed and the wash water discarded, c) mixed with salt, d) and packed in air-tight container.

Figure 2.8 a) Grilling *Amanita muscaria*, b) Mr. Satou enjoys a tasty morsel.

Figure 2.9 Yamabushi monk attempting to fly after consuming an unknown alcoholic beverage (after Earhart 1970)

2.4 DISCUSSION

A. COLLECTING

The young, budding *Amanita muscaria* appears completely white due to its complete universal veil. Collecting immature *A. muscaria* could lead to potentially fatal results as they appear identical to other immature amatoxin containing *Amanita* species (e.g. *Amanita verna, Amanita phalloides, Amanita virosa*). Amatoxins are lethal cyclic peptides that either inhibit DNA transcriptase II or cause specific damage to the liver.

B. DRYING

I found dried mushroom caps to be much more toxic than fresh or grilled caps due to the high concentration of muscimol present in dried caps (see chapter 3). Drying the mushrooms encourages the decarboxylation of ibotenic acid and results in the production of muscimol. While dried mushrooms are more toxic, the people of Sanada Town who consume dried *Amanita muscaria* in the manner described above do not complain of mushroom poisoning. Poisoning is dose dependent. Since the people are adding only a small amount of the dried mushrooms to rice dishes, they are not consuming enough of the dried caps to invoke any hallucinogenic effects.

C. PICKLING

Pickling *Amanita muscaria* is unique to this area of Japan and appears to be the most common method of preparation and consumption (Figure 2.5, 2.6). Informants said this pickling process is at least one century old but could date back to the 14th century with the construction of the salt road that led into the mountains of Nagano Prefecture. This

pickling process could not have been widespread before the salt road because there was no readily available salt source for this mountain village. When analyzing pickled mushrooms, I found no detectable amounts of either hallucinogenic compound. This suggests that pickled mushrooms are safe to eat (see chapter 3).

D. GRILLING

The men of Sanada Town who eat grilled mushroom caps tempt the dose dependency of the hallucinogenic compounds present in *A. muscaria*. They only consume a small amount and thus are able to enjoy the taste of the mushroom without inducing the hallucinogenic effects of the toxic compounds. When asked why they ate grilled *A. muscaria*, my informants compared the experience of eating a known poisonous mushroom like *A. muscaria* to eating *fugu*, the poisonous blowfish. The combined thrill of eating something poisonous and the outstanding taste makes this mushroom worth the risk.

When Gordon Wasson visited this area, he found he could not induce a hallucinogenic effect by consuming fresh *A. muscaria*. A Japanese friend suggested he grill the caps before eating them and this seemed to work much better (Wasson 1968). I found the fresh mushrooms to be only mildly toxic due to their large concentration of ibotenic acid and relatively small concentration of muscimol. Grilled mushrooms had a significantly higher amount of muscimol due to the decarboxylation of ibotenic acid (see chapter 3). Grilled mushrooms are thus more toxic and, as Wasson experienced, will induce hallucinations when several caps are consumed.

E. TINCTURE

While this method of consumption is rare, it may be the most interesting. It is possible that the Yamabushi monks have a similar practice of consuming an *Amanita muscaria* alcohol tincture in their *Goma* fire ritual. Earhart (1970) noted in his observations of the *Goma* ritual that these monks drank an alcoholic beverage that induced a flying sensation (Figure 2.9); however, he did not reveal the contents of the beverage. The *Goma* fire ritual of Japan may be a linguistic cognate of the *Soma* fire ritual of the Rig-Veda. If this is true, it may be possible to trace the origin of the Japanese *Goma* ritual, the tengu goblin, and the use of an *A. muscaria* tincture to the worship of *Soma* in the Rig-Veda.

2.5 ECONOMIC POTENTIAL

In Japan, each town or village has a specialty food for which it is famous. Tourists buy these specialty foods as souvenirs to take to their relatives so they may taste the unique flavors of that area. *Amanita muscaria* pickles have the economic potential to become the specialty food of Sanada Town. There are several problems hindering *A. muscaria* pickle production. First, few people continue to make this domestic product. People are beginning to forget how to do this traditional pickling process. The younger generations are not interested in learning how to detoxify poisonous mushrooms. Furthermore, the National Health Insurance Bureau of Japan openly discourages the consumption of *Amanita muscaria* in any form due to its known toxicity. If one wanted to sell *A. muscaria* pickles, there would be many legal issues to address before production began. Finally, birch forests cover only 2% of Sanada Town. While this produces enough *A.*

muscaria for the people of Sanada Town, it is not known whether this forest would support a commercial *A. muscaria*-pickling endeavor.

2.6 CONCLUSIONS

Amanita muscaria provides a unique food for the people of Sanada Town, Japan. The collection, preparation, and consumption of *A. muscaria* in this area of Japan are ancient traditions that people still practice today. In addition, it is possible that the Yamabushi monks use *A. muscaria* during their *Goma* rituals as a hallucinogen.

I found a substantial number of people who still practice the traditional methods of *Amanita muscaria* preparation. Pickling is the preferred method of preparation and consumption of *A. muscaria,* but some men enjoy consuming small quantities of grilled *A. muscaria* for its flavor and not necessarily for its hallucinogenic effects.

While this chapter gives a survey of *Amanita muscaria* use in Sanada Town, several important questions remain unanswered. More interviews are needed to better estimate total consumption levels among the population. Estimates of annual yield are needed if *Amanita muscaria* pickles are to become a local product. The efficacy of each method in removing the mushroom toxins is unknown. The next chapter addresses this question.

CHAPTER III. JAPANESE DETOXIFICATION OF BENI-TENGU-TAKE (*AMANITA MUSCARIA*)

3.1 INTRODUCTION

Wild harvested vegetables and mushrooms are extremely popular as foodstuffs in Japan. However, food poisoning from wild mushrooms including beni-tengu-take (*Amanita muscaria*) occurs every year (Ministry of Health and Welfare 1998). *A. muscaria* contains two hallucinogens - ibotenic acid and its decarboxylated derivative, muscimol (Bowden et al. 1965, Muller et al. 1965, Takemoto et al. 1964). Several cultures embrace the hallucinogenic effects these mushrooms produce (Lowry 1972; Wasson 1968; Saar 1991a, b). Some of America's counter-culture considers *A. muscaria* their narcotic of choice (Ott 1978).

Residents of the rural community of Sanada Town in Nagano Prefecture, Japan know *A. muscaria* is poisonous, yet these people continue to use beni-tengu-take in their local cuisine. These Japanese prepare *A. muscaria* in several ways. Most prefer to pickle the mushrooms while others grill, dry, or even drink a mushroom tincture. These people know, through oral tradition and personal experience, that all methods clearly are not equally effective in removing the hallucinogenic compounds (see chapter 2).

In this chapter, I provide scientific evidence to support the traditional Japanese practice of detoxifying beni-tengu-take. My study directly measures the efficacy of each preparation method employed by the people of Sanada Town. From my results, simple limits are suggested to minimize poisonings from consuming mushrooms prepared ineffectively.

3.2 MATERIALS AND METHODS

I used Gennaro et al.'s (1997) HPLC methods to quantify ibotenic acid and muscimol content in *Amanita muscaria* and its Japanese methods of preparation. These HPLC methods aided in determining the efficacy of each traditional method of preparation. I chose these methods because of their one-step extraction process and relative simplicity of the ion-interaction system.

A. CHEMICALS AND REAGENTS

I purchased octylamine, and phosphoric acid from Sigma Chemical Co. Sigma Chemical Co. provided ibotenic acid and muscimol standards. I used Fisher brand HPLC grade water for all solution preparations and HPLC grade acetonitrile to wash the column.

B. APPARATUS

I used a Shimadzu UV-2101PC UV/Vis spectrophotometer to determine absorption levels for Sigma standard solutions of unknown concentrations. A Hewlett Packard HP1090 coupled with a HP Chemstation determined retention times and peak areas of these standards in order to establish calibration curves for both hallucinogenic compounds. I then performed chromatographic analyses on all aqueous mushroom extracts. A diode-array detection (DAD) cell located within the HP 1090 HPLC recorded the absorbance spectra A/λ for mushroom extracts and standard solutions.

For pH measurements, I used a Corning 125 pH meter combined with its glass-calomel electrode. I performed sonication treatments using a probe Sonicator™ Cell Disruptor Model W-220F made by Heat Systems-Ultrasonics, Inc.

C. CHROMATOGRAPHIC CONDITIONS

I used an Alltech Allsphere ODS-2 (C18) (5μm, 250mm x 4.6mm) cartridge-type reverse-phase column coupled with an Alltech Allsphere ODS-2 (C18) (5μm, 7.5mm x 4.6mm) All-Guard™ Cartridge System guard pre-column for all rp-HPLC analyses.

The mobile phase was an isocratic aqueous solution of the ion-interaction reagents. I prepared the 5.0mM octylammonium o-phosphate aqueous solution at pH=6.4 by first adding the calculated amount of octylamine for the desired molar concentration to a known volume of HPLC grade water. I then buffered the 5.0mM octylammonium solution by adding concentrated phosphoric acid until I reached the desired pH of 6.4.

The chromatographic system was conditioned by passing the octylammonium o-phosphate mobile phase through the column until a stable baseline signal was obtained and standards of ibotenic acid and muscimol provided reproducible retention times. A minimum of twelve hours at a flow-rate of 0.5ml/min was necessary to condition the column. Samples were run in this isocratic aqueous system containing the octylamine ion-interaction reagent at a flow rate of 0.5ml/min. When not in use overnight, I programmed the HP1090 to maintain the flow-rate of the mobile phase at 0.1 ml/min to

keep the columns conditioned and allow for three continuous days of analyses. I washed the column and prepared new aqueous mobile phase after three days of use.

The column was washed by running water (0.5mL/min for 30 min), a 50/50 v/v water/acetonitrile mixture (0.5 ml/min for one hour), and finally acetonitrile (0.5 ml/min for 6 hours) through the HPLC system. This procedure was necessary to remove highly retained lipophilic matter that does not elute in aqueous mobile phase, but could affect column modification. I conducted a three-day wash periodically during which various gradients of acetonitrile and water were used to flush the system of all octylamine salts and eliminate any detrimental effects these reagents might have on the HPLC apparatus.

D. SAMPLE PREPARATION

1. COLLECTIONS

I collected bulk samples of *Amanita muscaria* in October 1999 in Sugadaira district of Sanada-town, Nagano Prefecture, in central Japan. The first bulk sample consisted of approximately seven kilograms of fresh *Amanita muscaria* (169 mushrooms). Mushrooms were dried at 50°C by two halogen shop-lamps.

I collected the second bulk sample of ca. 6kg of *Amanita muscaria* on the day before my departure for Florida International University. Once back in Sanada Town, I refrigerated this bulk sample at 4°C. These mushrooms remained at 4°C during transport to Florida

International University where they were then placed in a -20°C freezer until I could perform chemical analyses on each mushroom.

Key informants provided ample supply of pickled *Amanita muscaria* for later chemical analyses. I returned to Florida International University with 7 kilograms of pickled mushrooms. Pickled mushrooms provided by informants ranged from 2 weeks old to 10 years old.

2. SAMPLE EXTRACTIONS

I processed 5 to 15g sections of frozen fresh mushroom caps using a miniature food processor. Small sections were easier to filter later in the preparation process. I rinsed the processed pulp out of the food processor using a small amount of de-ionized water, put the processed pulp in a 50ml centrifugation tube, and sonicated the pulp for a total of 9 minutes to homogenize samples. The sonication probe had a tendency to overheat; therefore, the sonication was done in three, three-minute treatments. Between sonication treatments, I allowed the probe to cool for up to 5 minutes. After sonication, I separated the juice from the pulp by filtering through a Buckner funnel fitted with FisherBrand P8 qualitative filter paper. Vacuum pressure was used to speed up the filtering process. In order to improve the recovery of the hallucinogenic compounds, the pulp was washed twice with de-ionized water. I then diluted the juice up to 50mls and filtered a 1ml aliquot through a 0.22μm nylon membrane immediately before injection in the HPLC apparatus.

I pulverized 0.5g to 3.5g dried mushroom caps into a fine powder using the above-mentioned food processor. I then added roughly 20mls of de-ionized water to the powder and mixed thoroughly before sonication. The remainder of the sample preparation was done as mentioned above for fresh mushrooms.

To determine the effects of grilling the mushrooms, I grilled 10g samples of frozen mushroom caps over a Bunsen burner before subjecting these samples to the treatment mentioned above for fresh mushrooms.

Pickled mushroom caps were separated from their pickling juice and washed with de-ionized water for several hours prior to being subjected to the sonication and filtration treatments mentioned above for fresh mushrooms. I washed the mushroom to simulate the washing step performed the Japanese. The Japanese wash the pickled mushrooms to remove much of the salt from the pickles.

E. FRESH WEIGHT / DRY WEIGHT CONVERSIONS

As a means for comparison, I converted all sample weights to dry weight mmol/kg. While in the field, I collected and weighed approximately 200 mushroom caps and 200 mushroom stems. I numbered each mushroom's cap and stem, then dried them using a halogen-lamp driven mushroom drier at 50°C for 72 hours. Then, the dried mushrooms were vacuum-sealed in zip-lock bags with desiccant and brought them to Florida International University where I re-weighed these mushrooms to determine their dry-weight. I considered a sample dry when on three consecutive days the sample gave a

consistent weight. Linear regression was used to determine the correlation coefficient between fresh weight and dry weight of the above-mentioned 200 mushrooms. This model was then used to convert all other fresh-weight measurements into dry-weight concentrations for final comparisons.

F. CALIBRATION CURVES

I made several dilutions of ibotenic acid and muscimol Sigma standards, then used UV spectroscopy to determine the concentration of each dilution. I used at least three injections of 5, 10, and 15μL aliquots for each diluted Sigma standard. I calculated concentrations for ibotenic acid and muscimol standards by using their extinction coefficients. The extinction coefficient for ibotenic acid at 230nm (pH 7) is 2420 while muscimol has an extinction coefficient of 2810 at 230nm (pH 7) (Eugster 1969). I used linear regression to analyze this data and the line of best fit to determine concentrations for the crude mushroom extracts. The calibration curves established for ibotenic acid in the concentration range between 12.0 mmol/kg and 0.03 mmol/kg showed a good correlation between peak area and concentration. Likewise, calibration curves for muscimol in the concentration range between 15.2 mmol/kg and 0.0 2mmol/Kg showed a good correlation between peak area and concentration. The plots could be fitted by straight lines with correlation coefficients always higher than 0.995. Detection levels as low as 0.02 mmol/kg and 0.03 mmol/kg were respectively evaluated for muscimol and ibotenic acid. I updated calibration curves weekly to ensure accurate calculations.

G. STATISTICAL ANALYSIS

I used independent-samples t-tests to compare mean ibotenic acid and muscimol concentrations in mushroom caps within each treatment. A one-way analysis of variance (ANOVA) was used to compare ibotenic acid and muscimol concentrations independently between treatments. In addition, I ran an ANOVA to compare total mean hallucinogenic compounds (ibotenic acid plus muscimol) detected between treatments. I calculated means and standard errors for ibotenic acid and muscimol concentrations using descriptive statistics. I distinguished treatments into homogenous subsets using Tukey's post-hoc analysis. All statistical analyses were executed using univariate analyses in SPSS base 9.0 (SPSS software, Chicago, IL.).

3.3 RESULTS

A. EXTRACTION

Aqueous mushroom extracts are unstable and contain many lipophilic (oily) substances. If I did not run samples immediately after extraction, precipitates fell out of solution within my samples. I also noticed fungal growth in samples that were 24 hours old at room temperature or three days old at 48C. Due to this inherent instability of the aqueous extracts, I did not replicate samples to test for inter-day repeatability.

B. HPLC

The HPLC system I used produced good base-line separation, resolution and retention between ibotenic acid and muscimol standards (Figure 3.1). To ensure that peaks for ibotenic acid and muscimol were present in a mushroom extract chromatogram, I always

checked the UV spectra for both compounds and compared them with published spectra. In addition, I spiked mushroom extracts with standards to verify peak identity and determine the recovery of ibotenic acid and muscimol.

C. TREATMENTS

The HPLC system produced good base-line separation, resolution, and retention between ibotenic acid and muscimol in extracts from each of the four treatments. Figure 3.2 shows a representative chromatogram for each treatment. I quantified ibotenic acid and muscimol by using the peak area of both compounds at a wavelength of 230nm to calculate their respective concentrations.

Visual inspection of the means shows higher concentrations of hallucinogenic compounds in caps than in stems for all treatments (Table 3.1). I analyzed only three stems for each treatment and therefore did not perform statistical analyses between caps and stems. Stem mean concentrations are included in Table 3.1 for reference only.

1. FRESH MUSHROOMS

I performed HPLC analyses on fresh mushrooms as a means of comparing the three different traditional methods of preparation: grilling, pickling, and drying. There is a significantly higher mean ibotenic acid concentration than mean muscimol concentration in fresh mushroom caps, t (62) =10.88, p<.05 (Figure 3.3, 3.4). As shown in Table 3.1, fresh mushroom caps have a significantly higher mean ibotenic acid concentration than grilled, dried or pickled mushrooms. As can also be seen, the mean amount of muscimol

found in fresh mushroom caps does not differ significantly from grilled or pickled mushroom caps (Figure 3.3).

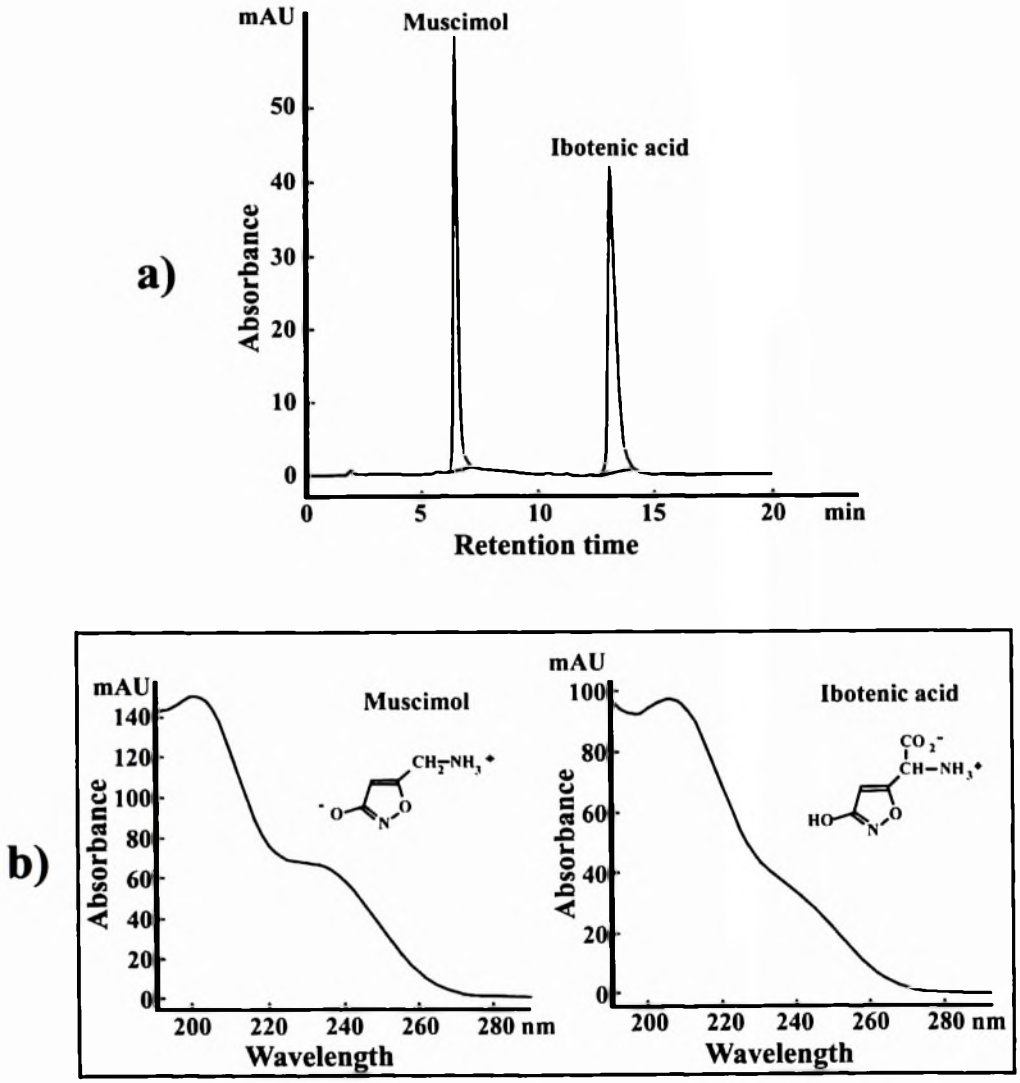

Figure 3.1 a) Chromatogram of ibotenic acid and muscimol standards in aqueous solution showing relative retention times,
b) Absorption spectra of muscimol and ibotenic acid in H_2O

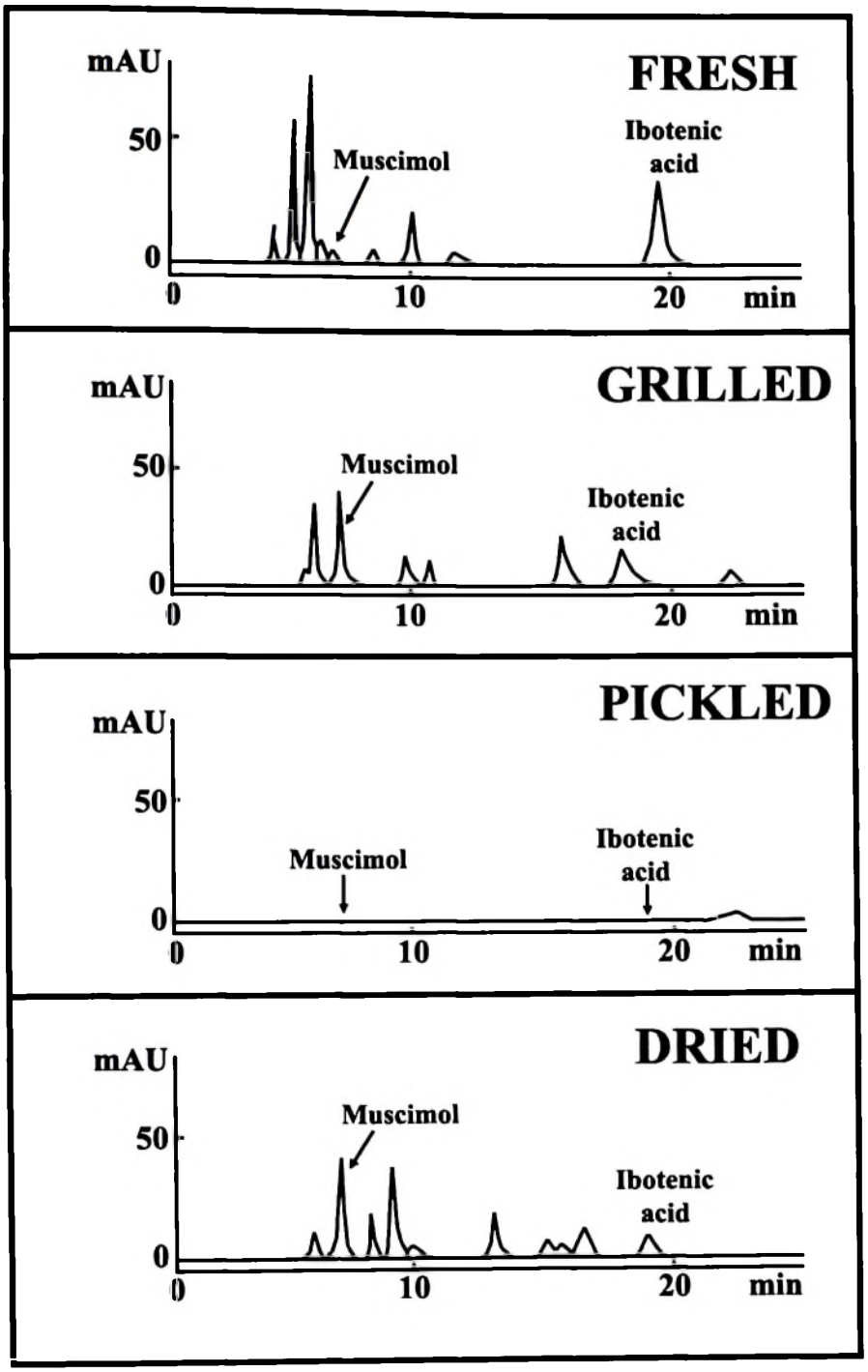

Figure 3.2 Representative chromatograms of *Amanita muscaria* caps at 230nm from each treatment showing relative peak areas for muscimol and ibotenic acid

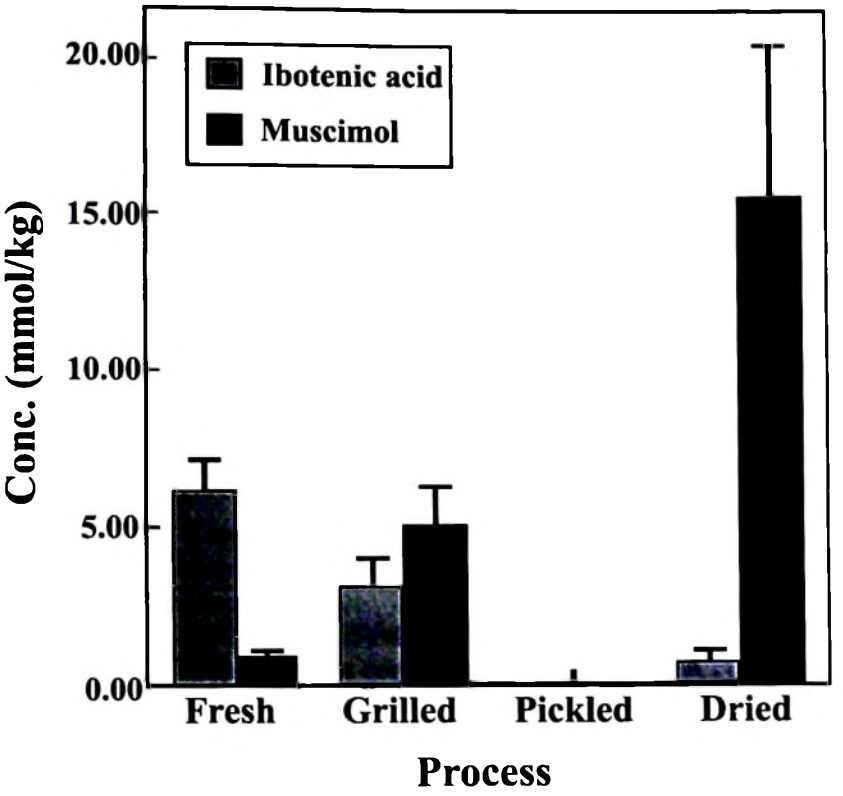

Figure 3.3 Mean ibotenic acid and muscimol concentrations in *Amanita muscaria* caps (Error bars = 95% CI). Note: Concentration for pickled is ND.

Table 3.1 Mean and standard error of hallucinogenic compound (ibotenic acid and muscimol) concentrations in *Amanita muscaria* fruiting bodies

Treatment		Mean Ibotenic acid concentration (mmol/kg)	Mean Muscimol concentration (mmol/kg)	Total Mean Hallucinogenic Compounds (mmol/kg)
Fresh mushrooms	Caps	6.17 (0.47)[a]	0.93 (0.08)[ab]	7.11 (0.49)[a]
	Stems	2.89 (0.43)	0.51 (0.17)	3.40 (0.59)
Grilled mushrooms	Caps	3.17 (0.39)[b]	5.17 (0.53)[b]	8.34 (0.88)[a]
	Stems	2.02 (0.29)	.29 (0.08)	2.31 (0.33)
Pickled mushrooms	Caps	ND[c]	ND[a]	ND[b]
	Stems	ND	ND	ND
Dried mushrooms	Caps	0.72 (0.19)[c]	15.66 (2.31)[c]	16.38 (2.34)[c]
	stems	0.14 (0.03)	4.33 (1.84)	4.47 (1.09)

Note: ND = None Detected

Ibotenic acid in caps: ANOVA $F(3,65)=41.5$, $p<.05$. Muscimol in caps: ANOVA $F(3,65)=42.6$, $p<.05$. Hallucinogenic compounds in caps: ANOVA $F(3,65)=23.51$, $p<.05$. The results of Tukey's post-hoc test are indicated with superscript letters. Items with different superscript letters differ significantly at the .05 level. ( )=SE

2. DRIED MUSHROOMS

As shown in Table 3.1, the total amount of hallucinogenic compounds is significantly higher in dried mushrooms than all other treatments. Dried mushroom caps contain a significantly higher amount of muscimol than ibotenic acid, t (30)=6.44, p<.05 (Figure 3.3). The mean concentrations of muscimol in dried mushroom caps differed significantly from fresh, grilled, and pickled mushroom caps (Table 3.1). However, the mean ibotenic acid concentration found in dried mushroom caps did not differ significantly from that found in pickled mushroom caps (Table 3.1).

3. GRILLED MUSHROOMS

Grilled mushroom caps contain a significantly higher mean concentration of muscimol than ibotenic acid, t (20)=3.04, p<.05 (Figure 3.3). The total amount of hallucinogenic compounds present in caps does not differ significantly between fresh and grilled mushrooms (Table 3.1). The mean ibotenic acid concentration found in grilled mushroom caps was significantly lower than that found in fresh mushroom caps and significantly higher than that found in dried and pickled mushroom caps (Table 3.1). The mean muscimol concentration found in grilled mushroom caps was much higher than but did not differ statistically from fresh mushroom caps. However, the mean muscimol concentration found in grilled mushroom caps was significantly higher than that found in pickled mushroom caps and significantly lower than that found in dried mushroom caps (Table 3.1).

4. PICKLED MUSHROOMS

Both ibotenic acid and muscimol are below quantifiable levels in pickled mushroom caps and stems. The mean ibotenic acid concentration found in dried mushroom caps did not differ significantly from the mean ibotenic acid concentration found in pickled mushroom caps. Likewise, the mean muscimol concentration found in fresh mushroom caps did not differ significantly from the mean muscimol concentration found in pickled mushroom caps.

3.4 DISCUSSION

I found fresh mushrooms to contain low concentrations of muscimol and high concentrations of ibotenic acid. This finding supports other research that suggest ibotenic acid is the only hallucinogenic compound present *in situ* (Chilton 1994, Gennaro et al. 1997, Tsunoda et al. 1993) and that decarboxylation occurs once the mushrooms are picked, handled, or eaten.

Gennaro et al.'s (1997) study found mean concentrations of 6.3mmol/kg and 1.45mmol/kg ibotenic acid in caps and stems respectively and 3.33mmol/kg and 0.70mmol/kg muscimol in caps and stems respectively using the same methods employed in my study. The results of this study are comparable despite the different geographic origin of the samples. Gennaro et al.'s (1997) higher mean muscimol concentrations may be due to her integrating peak area and peak height at two wavelengths. Gennaro et al. (1997) reports no significant difference observed at the two wavelengths used for ibotenic acid. Thus, our results are quite similar. However, sensitivity for muscimol is much

lower at 254nm than at 230nm. I chose to use only the peak area at 230nm because I found both compounds to absorb well at this wavelength. Had I integrated peak height and peak area at both wavelengths, my quantification of muscimol concentrations might be higher.

Conversely, Tsunoda's (1993) study found mean concentrations of 2.3mmol/kg ibotenic acid and 0.20mmol/kg muscimol in fresh, frozen, and freeze-dried *A. muscaria* samples collected in Nagano Prefecture. While both Tsunoda and I collected from Nagano Prefecture, my mean concentrations are higher than those reported in Tsunoda's study. This discrepancy may be due in part to differences in methodology. It is unclear as to whether Tsunoda homogenized whole mushrooms in his study or if the above measures are concentrations found in caps only. Tsunoda's methods of extraction are much more involved. With each additional step in the extraction process, one increases the risk of sacrificing total recovery. Tsunoda's ion-pairing HPLC system requires the use of SDS detergent and an extreme pH (pH 2.2). This low pH is detrimental to the column and the HPLC system. Tsunoda neglects to mention these effects in his article. Environmental factors also may help explain this discrepancy. Tsunoda collected *A. muscaria* during the summer of 1987. Environmental conditions, location, season, and individual mushroom specimens affect chemical variations in *A. muscaria* (Hall and Hall 1994, Spoerke and Rumack 1992, Repke et al 1978, Eugster 1967). It may be possible that chemical variation exists between fruiting seasons or that a season's climate may cause fluctuation in ibotenic acid production for that particular season.

The total amount of hallucinogenic compounds present in fresh and grilled mushrooms did not differ. However, one must look at the ratio of these two hallucinogenic compounds and consider how ibotenic acid might react to the grilling process in order to determine the total toxicity of grilled mushrooms. Grilling the caps increases the rate of decarboxylation of ibotenic acid into muscimol and increases the toxicity of the caps.

3.5 CONCLUSIONS

In this chapter, I presented results on concentrations of ibotenic acid and muscimol present in fresh, dried, grilled, and pickled *Amanita muscaria*. I showed fresh *A. muscaria* to contain an average of 9.06 mmol/kg of ibotenic acid and 1.44 mmol/kg of muscimol. These findings agreed with other investigators who used ion-interaction HPLC (Gennaro et al. 1994) but were slightly higher than the findings of investigators who used ion-pairing HPLC (Tsunoda et al. 1993).

I showed that dried *Amanita muscaria* contains considerable amounts of muscimol and are quite toxic. The culinary use of dried *A. muscaria* should be discouraged. Dried *A. muscaria* can safely be used if they are re-hydrated and pickled.

In addition, I showed that grilling *A. muscaria* actually increases the toxicity of the mushrooms by encouraging the decarboxylation of ibotenic acid into muscimol. Due to the variable concentrations of both hallucinogenic compounds and the variable rate of decarboxylation, one can never be sure how much muscimol is present in a grilled

mushroom. Generally, the longer the mushroom has been on the grill, the more muscimol it may contain. The consumption of grilled *A. muscaria* should be discouraged.

This study showed that pickled mushrooms are safe for human consumption. I tested pickled mushrooms that were from 2 weeks old to ten years old and found no detectable ibotenic acid or muscimol in any pickled mushroom samples. The practice of pickling *A. muscaria* should not be discouraged; however, it is important the proper pickling procedures be made available to those who might like to try this traditional Japanese dish.

CHAPTER IV. CONCLUSIONS

The primary objectives of my research were to document accurately the use of *Amanita muscaria* in this region of Japan and to provide quantitative scientific data to support the local methods of detoxification. I have arranged my data into two categories: ethnomycological and biochemical. Overall conclusions are listed by category in the following pages.

Ethnomycological data:

Who eats *Amanita muscaria*?

- A large portion of the population of Sanada Town consumes *Amanita muscaria*. Interviews conducted in neighboring villages revealed few persons who knew anything of *A. muscaria* preparation and consumption. *A. muscaria* consumption appears to be a local tradition.

- The consumption of *A. muscaria* is more popular with the older generations in Sanada Town.

- More men eat grilled *A. muscaria* than women do.

How is *A. muscaria* prepared?

- Pickling is the preferred method of preparation. Pickled *A. muscaria* are prepared by boiling the mushrooms for 5 to 15 minutes, washing thoroughly in running water, then packing the blanched mushrooms in salt. These salted pickles are allowed to stand for at least one month before being consumed.

- People also grill fresh *A. muscaria* over an open fire.
- Dried *A. muscaria* is occasionally used as a seasoning. Small flakes are sprinkled into rice dishes to enhance their flavor. Dried mushrooms may also be re-hydrated and pickled by following the prescribed pickling procedure for fresh mushrooms.

- Very few people consume an *A. muscaria* tincture that is made of mushrooms soaked in alcohol. This practice may have been part of a ritual performed by the Yamabushi monks.

How much and how often are these mushrooms consumed?

- Pickled *A. muscaria* are eaten in small quantities as a side dish or hors d'oeuvre as part of New Year festivities. The pickling process usually is performed only once a year.

- Men usually consume a small quantity of grilled mushrooms once a year. Many are superstitious and believe the toxins ingested remain in the body and begin to accumulate over the years. Most feel it is best not to overindulge. People seem to know their limits from personal experience.

- People rarely consume dried mushrooms or *A. muscaria* tinctures.

Why is *A. muscaria* eaten?

- Most Japanese eat *A. muscaria* for its unique taste and not for its hallucinogenic effects.

Biochemical data:

How effective are traditional methods in detoxifying *A. muscaria*?

- All traditional methods of detoxifying *A. muscaria* are not equally effective. Pickling is very effective in removing both ibotenic acid and muscimol. Grilling and drying increase the toxicity of *A. muscaria* by encouraging the decarboxylation of ibotenic acid and increasing the concentration of muscimol.

When are *A. muscaria* rendered safe to eat?

- The boiling step of the pickling process removes most of the ibotenic acid and muscimol present in fresh mushroom tissue. Pickled *A. muscaria* are the only mushrooms guaranteed safe for human consumption.

RECOMMENDATIONS

For future research, I recommend that:

- traditional uses of *Amanita pantherina* be documented through informant interviews.
- researchers investigate the possible use of *Amanita muscaria* tinctures by yamabushi monks in the *Goma* fire ritual.
- linguists determine whether *Goma* practiced by yamabushi monks in Japan and *Soma* of the Rig-Veda are cognates.
- traditional Japanese detoxification methods for other poisonous mushrooms be documented and the mode of detoxification be identified through biochemical analyses.

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