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A Review: Effect of Ozone on Milkweeds (*Asclepias* spp.) in USA and Potential Implications for Monarch Butterflies

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Abstract

Ozone is the most important phytotoxic air pollutants in the U.S., in both agricultural and ecological settings. Common milkweed (*Asclepias syriaca* L.) isone of the most important ozone-sensitive, native, bioindicatorplants in the U.S that is capable of detecting phytotoxic levels of ambient ozone. Diagnostic ozone-induced symptoms on milkweed foliage are brown to black spots ("stipples") on the axial leaf surface, in addition to prematuredefoliation (accelerated senescence). Other*Asclepias* spp.in the U.S.might also serve asozone-sensitive bioindicators, butfew have been tested.We suggest testing them, and also suggest that ozone injury to milkweedsmig htinfluence the monarch butterfly (*Danaus plexippus* L.) life cycle, since adult female monarchsoviposit only onmilkweed leaves and resulting caterpillars feed exclusively on milkweed leaves. We hope this review will stimulate new research and provide a framework for further studies in the complicated, interwoven, tripartite interaction among ambient ozone, milkweeds, and monarch butterflies. This review discusses sources, concentrations, and dispersion of the air pollutant ozone in the U.S.; the time frame for the historical development of ozone-sensitive bioindicator plants; and the lack of data regarding ozone-sensitivity among milkweed species in the U.S.

Keywords: Air pollution, *Asclepias*, Bioindicators, Butterflies, Milkweeds, Ozone

1. Ozone as an Air Pollutant

Ozone (O_3) is a triatomic oxygen molecule present throughout the earth's atmosphere that is considered either beneficial or harmful, depending on its location. Beneficial ozone occurs at~10–50 km above the earth's surface, where it absorbs potentially damaging ultraviolet radiation. In contrast, tropospheric ozone is located from ground level to ~10 km where it is an air pollutant that is harmful to human health, plants, and terrestrial ecosystems (USEPA 2003, 2013).

In the early 1950s, the atmospheric chemistry of the infamous Los Angeles (LA) photochemical "smog" was characterized, and ozone identified as a major component of smog. Haagen-Smit (1952) and Haagen-Smit et al. (1952) reported that ozone is formed during the day when the chemical precursors to ozone formation (nitrous oxides [NOx] and volatile organic compounds [VOCs]) react in sunlight-driven atmospheric photochemical reactions. These authors, as well as the Stanford Research Institute (SRI 1954), reported that common sources of NOx and VOCs included fossil fuel combustion, vehicle emissions, industrial processes, and others. During the next 60 years, thousands of research papers were published characterizing ozone as a tropospheric air pollutant (Krupa 1997, Krupa et al. 2001).Papers also revealed that high levels of ozone occurred in rural areas far downwind from precursor sources where they were capable of injuring agricultural crops and native ecosystems (Krupa and Manning 1998).

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Background ozone concentrations (those not influenced by local anthropogenic precursors) in the Northern Hemisphere peak during spring, usually in May, with mean annual background levels ranging from 20–45 ppb (Vingarzan 2004). Ambient ozone concentrations near and downwind from pollution sources often peak later in the summer and may exceed 60 ppb in northeastern U.S. (NE) (Krupa 1997, Krupa and Manning 1988). Atmospheric levels of combined anthropogenic-generated and background ozone vary daily and seasonally in the U.S. Daily variations typically follow diurnal day-night patterns, since sunlight drives the photochemical reactions that produce ozone. Ozone concentrations are generally greatest in the late afternoon, when production exceeds continuous destruction via the reverse reaction, and lowest in early morning, when NOx is present to react with and consume ozone. Seasonally, ozone formation is often greater in the summer due to longer day lengths and greater solar angle of incidence (resulting in greater light intensities), as well as higher temperatures that result in higher volatilization rates of chemical precursors (Krupa et al. 2001). One measurement used to relate such seasonal levels of ambient ozone to ozone-induced plant injury is the SUM60 metric. The metric often used is the sum of the number of hours from 8:00 AM to 5:59 PM that contain $\geq$ 60 ppb ozone during a 90-day period. In a series of ozone-injury surveys for the U.S. Fish & Wildlife Service, Davis (2007 a,b; 2009; 2011) and Davis and Orendovici (2006) used a SUM60 ozone metric to characterize the seasonal pattern of ozone-injury to vegetation.

Elevation also influences variations in tropospheric ozone concentrations. At higher elevations, diurnal ozone concentrations may remain relatively constant, resulting in increased nighttime levels relative to lower elevations, where ozone is destroyed at night via reaction with NOx (Krupa et al. 2001). Neufeld et al. (1992) reported that ozone concentrations at higher elevations in the Great Smoky Mountains National Park (GSMNP), on the Tennessee-North Carolina border, exceeded those observed at lower elevations. Likewise, Orendovici-Best et al. (2010) reported that ozone concentrations in forested north-central Pennsylvania were significantly and positively correlated with elevation, leading to their conclusions that forests in this perceived "pristine" location may be at risk from ozone.

Seasonal ozone concentrations follow an unusual pattern in the Uintah Basin in Utah and the Upper Green River Basin in Wyoming, U.S. (Utah State University 2017, available on-line). These basins trap ozone-precursors generated by extensive gas and oil drilling activities. Seasonal ozone concentrations in these localized areas are greatest during the winter (e.g. January and February) when the ground is snow-covered, and if low winds, intense sunlight, and temperature inversions occur. In these basins, similar to oil and gas drilling areas in the NE, wintertime peaks of ozone might pose human health problems, but are unlikely to cause plant injury since most plants are dormant during winter months. However, such drilling activities may also contribute precursors that form smaller ozone peaks during the summer (Utah State University 2017, available on-line),possibly causing injury to ozone-sensitive plants. The recent documentation of this wintertime phenomenon illustrates that local industrial activities can produce the precursors that form local ground-level ozone, which may become trapped within basins. Interestingly, natural gas drilling activities in Marcellus Shale Basin in the NEgenerate significant emissions from both conventional and unconventional wells (Omara et al. 2016), including ozone-forming precursors such as VOCs (Swarthout et al. 2015). Other shale formations in the NE also contain extensive gas and oil deposits (Roen 1984), where associated drilling and transport activities may generate ozone precursors (Olaguer 2012). This phenomenon is greatly under-studied.

On 1 October 2015, the U.S. Environmental Protection Agency (EPA) lowered (strengthened) the primary and secondary U.S. National Ambient Air Quality Standard (NAAQS) for ozone from 75 to 70 ppb (USEPA 2015a,b). The 70-ppb ozone NAAQS is based on the 4th highest daily maximum ozone concentration, averaged across 3 consecutive years for an averaging time of 8 hours, not to exceed 70 ppb to avoid a violation. The reduction makes the new ozone NAAQS more stringent to help protect public health and welfare, as well as to protect the health of plants and terrestrial ecosystems(USEPA 2015a,b). Figure 1A,B illustrates downward trends in U.S. ambient ozone levels during two time periods, as compared to 70 ppb ozone (horizontal dashed line), the ozone concentration used in calculating the ozone NAAQS (https://www.epa.gov/air-trends/ozone-trends).Figure 1A,B reveal 32 and 22% (data calculations not shown) declines in U.S. ambient zone levels during the respective time frames.

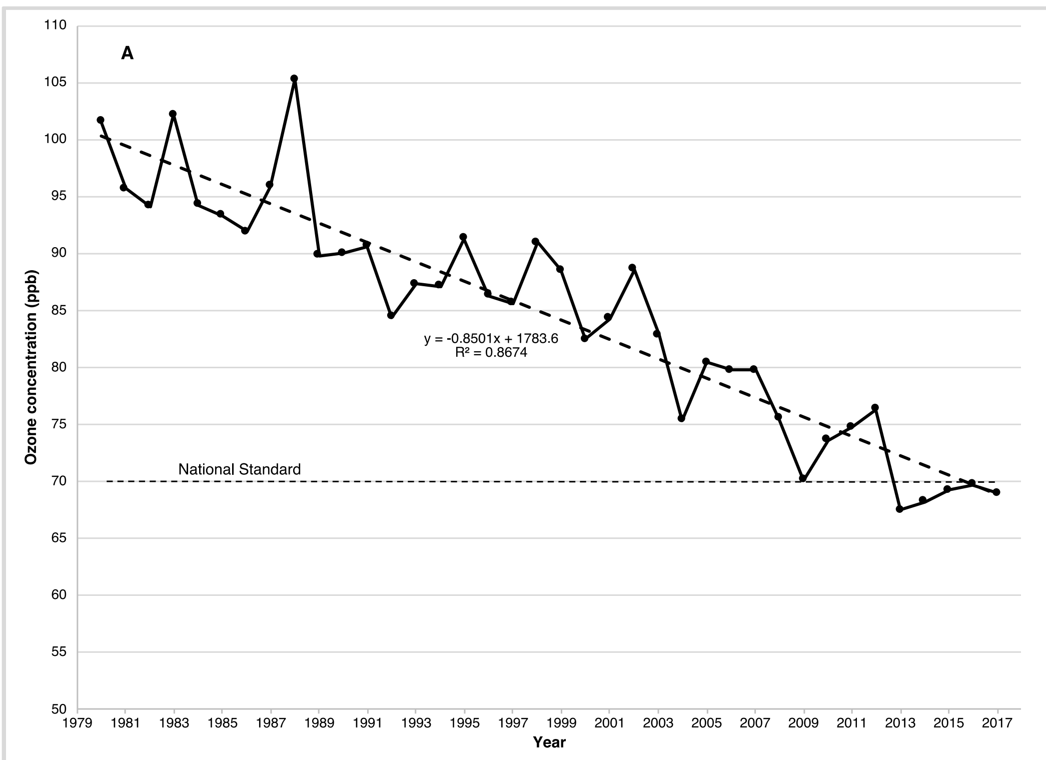
2. Development of Ozone-sensitive Broad-leaved Bioindicators

2.1. Historical Timeline

English botanists studying the effects of electrical discharges on plant respiration in the laboratory were the first to report possible ozone injury to plants (Knight and Priestly 1914). As a side-effect of the electrical discharges, the researchers reported that "…chlorophyll in the upper portions [of the plants] had been bleached by the oxidizing products produced in the atmosphere by the discharge."

Plant species used in the experiments included Brussels sprouts, pea, rye, and wheat. Unfortunately, the authors did not report varieties, species impacted, or identify the atmospheric agent responsible for the bleaching, although it was later assumed that the bleaching was likely caused by ozone formed during the electrical discharges (Homan 1937).

In 1937, Homan reported building a state-of-the art laboratory ozone-exposure chamber in which he subjected bean plants to various ozone concentrations and durations. He carefully controlled and monitored environmental conditions during the ozone exposures, including air flow, light, temperature, and soil conditions, as well as plant factors including plant size, number, and vigor. Homan was the first to record and report such environmental conditions during ozone exposures as well as being the first researcher to document and report the lowest ozone concentrations and exposure times needed to cause visible plant injury. Furthermore, Homan was the first to describe a variety of ozone-induced plant symptoms that included leaf bleaching, yellowing, speckling, brown necrotic spots, and wilting, as well as plant stunting and reduced plant dry weight. He also noted that ozone-induced symptoms were most severe on the oldest leaves, that the youngest leaves were not affected, and that larger veins on affected leaves typically remained green. Homan also suggested that ozone, as a strong oxidizing agent, may accelerate the plant aging process and hypothesized that the "…physiological action of ozone is partly the saturation of lipoid compounds in the cell membranes, resulting in loss of semi permeability, wilting, oxidation of cell contents, and death of the cell." His findings were the first detailed, published descriptions regarding symptoms caused by ozone, which later led to the first accurate identification of ozone-sensitive bioindicator plants.



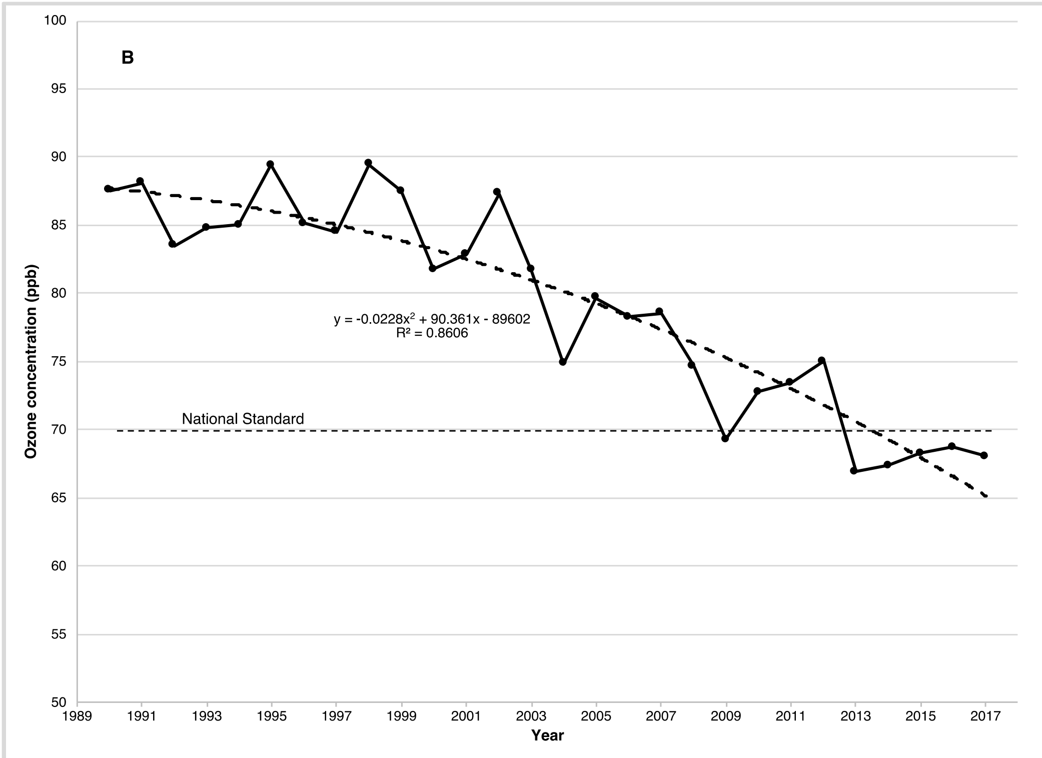


Figure 1A,B.Downward trends of average U.S. ambient ozone concentrations (ppb) during two time periods. Figure 1A illustrates the national ozone trend from 1980-2017, based on data from 200 ozone monitoring sites. Figure 1B illustrates the more recent national trend from 1990-2017, based on data from 422 ozone monitoring sites. Best fit (R^2) for the dashed trend line in Figure 1A was linear, whereas the best fit of the curved trend line in Figure 1B was a 2nd order polynomial. The horizontal dashed line depicts70 ppb ozone, the value used in calculating the current U.S. NAAQS for ozone. Data available online at https://www.epa.gov/air-trends/ozone-trends; accessed 9 August 2018.

During the 1950s, research regarding the formation of tropospheric ozone, as well as its phytotoxic effects, dramatically increased, especially within the LA Air Basin. Middleton et al. (1950) described visible injury to herbaceous plants due to the LA photochemical smog, which included ozone. Two years later Haagen-Smit et al. (1952) reported ozone injury on leaves of alfalfa, endive, and spinach plants following laboratory exposures to the photochemical oxidants in LA smog. Soon after, Middleton (1956) observed ozone injury on field beans in California. These early studies helped confirmed that ozone was a major phytotoxic component of photochemical smog on the West Coast of the U.S.

In a seminal paper, Richards et al. (1958) presented a detailed description of ozone-induced injury to the foliage of table and wine grapes that had occurred as early as 1954 in California. They reported that the first visible symptoms on ozone-injured grape leaves were small, discrete, brown to black, punctuate spots on the upper leaf surface, later termed "stipples". The authors noted that grape stipple was comprised of brown to black groups of pigmented palisade mesophyll cells, which because of their dark color, were visible though the hyaline upper epidermis and overlying cuticle. Richards and colleagues observed that ozone-induced grape stipple first appeared on young, fully expanded leaves, but was absent on the very youngest foliage. As new stippling occurred on developing grape leaves, a progressive accumulation of stipple was noted on the older foliage. Secondary symptoms followed the formation and accumulation of stipple, including leaf bronzing, yellowing, and premature senescence/defoliation. These important descriptions by Richards and colleagues further allowed recognition of visible ozone injury on many broadleaved plants across the U.S., leading to later development and use of many ozone-sensitive bioindicators.

In 1959, Heggestad and Middleton reported ozone injury on field-grown tobacco in eastern U.S. In the same year, Ledbetter et al. (1959) reported injury on 32 plant species and varieties following exposure to ozone within controlled environment chambers in a greenhouse.

Ozone-induced foliar symptoms again included dark stipple, light flecks, necrotic patches, and general chlorosis. Soon after, Hill et al. (1961) exposed 34 plant species to ozone in a controlled-atmosphere greenhouse in Utah. The results of Hill and colleagues confirmed many of the symptom descriptions previously reported by Richards and his colleagues, including: adaxial leaf stipple was a common response to ozone among many broadleaved plant species; when present, palisade cells were more readily injured by ozone than spongy mesophyll cells; ozone-sensitivity increased with maturity of leaf tissue; young leaves were only injured at the tips; leaf veins generally remained free of stipple; and within the leaf lamina, most stipple was located close to the vein edges. These morphological descriptions again allowed for further identification and confirmation of symptoms on additional ozone-sensitive bioindicator plants.

Following these extensive morphological descriptions of ozone injury to plants that formed the foundation for identification and development of ozone-sensitive bioindicators, researchers began to study physiological effects of ozone. In an important early paper, Howell (1974) stated that chloroplast phenols, as well as enzymes capable of oxidizing phenols, were compartmentalized and separated from each other by membranes. However, intracellular ozone, or its oxidized products, injured the surrounding membranes and caused them to leak. The enzymes could then oxidize phenols to quinones, which in turn, could polymerize amino acids, amines, and sulfhydryl groups to form the reddish-brown pigments (stipples) that researchers had observed on leaves exposed to ozone. Howell further stated that the polymers, such as oxidized phenols, formed in ozone-injured leaves might affect the nutritional value of ozone-injured foliage. This observation had later relevance when it was found that some leaf-chewing insects such as monarch butterflies preferentially fed on host foliage that had been stippled by ozone (e.g., was high in apparently nutritious phenolic compounds) (Bernays and Woodhead 1982, Bernays et al.1983). During the century since the initial report of Knight and Priestly (1914), numerous papers dealing with the effects of ozone on plants have been published, ranging from physiology (Amundson et al. 1986, Reich and Amundson 1985);ecology (Barbo et al. 1998); to general risk assessment (Kohut 2007). This body of papershelped confirm that ozone is the most important phytotoxic air pollutant in the U.S. (Krupa and Manning 1988) and the world (Krupa et al. 2001). Ozone-induced morphological injuries were further characterized and illustrated for numerous broadleaved plants, allowing prospective use of a wide number ozone-sensitive bioindicator plants (Skelly 2000, Skelly et al. 1987, USDOI 2003).

2.2. Use ofOzone-sensitive Bioindicator Plants

Useful ozone bioindicatorsare ozone-sensitive plants that produce characteristic leaf symptoms following uptake of phytotoxic levels of ambient ozone (Manning and Feder 1980;Smith et al. 2003, 2007). Bioindicators can be used during field surveys to indicate that phytotoxic levels of ambient ozone are/were present, assuming environmental conditions were conducive for ozone uptake and ozone-inducedplant injury (Krupa and Manning 1988; Manning and Feder1980; Smith et al. 2003, 2007).To be useful in field surveys, bioindicator plantsmust be sensitive to ozone,respond to ozone in predictable and reliable ways, be widespread within the area of interest, and be easy to identify by field personnel (Manning and Feder 1980; Smithet al. 2003, 2007).Bioindicator plants offer an alternative to chemical or electrical air monitors that directly measure ambient pollutant levels. Plants can be used to detect phytotoxic levels of ozone in areas where analytical equipment cannot be utilized due to expense, lack of electricity, or unavailability of instruments. Although bioindicator plants do not provide ozonedata (concentrations), they offer flexibility in changing size and location of monitoring plots. Also, as living organisms, bioindicator plant responses may be better correlated with stress from ozone, as compared to data from chemical/mechanical monitors (Smith et al. 2003, 2007).

Summary tables have been published that list ozone-sensitive plant species that are potentially useful asozone bioindicators (e.g., USDOI 2003). Ozone-sensitivity categoriesin such tables areoften somewhat subjective, including sensitivity categories as such as “not injured (tolerant), light-injury, moderate-injury, and severe-injury” that are usually based on observed levels of leaf stipple. Such injury classifications are often derived from three main types of studies: i) ambient ozone exposures in field open-top chambers (Barbo et al. 1998, Kohut et al. 2000, Neufeld et al. 1992); ii) field surveys to detect ambient ozone injury on plants growing in their native habitat (Chappelka et al. 1997, Eckert et al. 1999, Skelly et al. 1987, Smith et al. 2003, Temple 1999, Treshow and Stewart 1973); and iii) exposure to ozone in greenhouse exposure chambers, such as Continuous Stirred Tank Reactor (CSTR) (Heck et al.1978)chambers in greenhouses (Kline et al. 2008, 2009; Myers et al. 2018; Orendovici et al. 2003). Ozone-sensitivity rankings based on field exposures to ozoneperhaps yield the most realistic sensitivity classifications, especially if replicated by trained observers over several years.

However, Orendovici et al. (2003) stated that ozone-sensitivity ratings derived from ozone exposures in controlled-environment chamber studies are also useful if: i) similar symptoms do not occur on non-exposed control plants; ii) there are clear dose-response relationships; and iii) symptoms are similar to those described in the literature. Even if derived from different types of studies, the subjective classification of ozone-sensitive bioindicator species may yield useful clues to help identify new bioindicator species.

2.2.1.Milkweeds(*Asclepias* spp.) asBioindicators.

Asclepias spp. are classified within the dogbane family (Apocynaceae), milkweed subfamily (Asclepidiaceae). Woodson (1941, 1954)listed~105 indigenous milkweed species in North America. Fishbein et al. (2011) and the USDA (2017) reported~75-76 milkweed speciesnative to the U.S., not counting subspecies and synonyms. More recently, Agrawal (2017) estimated that the numbers of milkweed species in the Americas number ~130. However, most milkweed species found in the U.S.have not been evaluated for ozone-sensitivity. Of the 75 species listed in Table 1, including the non-native tropical milkweed (*A. curassavica*), which is from Mexico but commonly planted in the U.S., only 17 (23%) have been rated for ozone-sensitivity.

Common milkweed (*A. syriaca*) is one of the most widely used, ozone-sensitive, bioindicator plants in the U.S. The species' high sensitivity to ozone, based on visual injury, was first reported by Duchelle and Skelly (1981), using open-top chambers in which milkweed plants were exposed to ambient ozone in the field. The authors characterized ozone-induced injury on common milkweed as dark purple-to-black stippling on the upper surface of the lower, older leaves, as well as premature defoliation. Since the Duchelle and Skelly report, commonmilkweed has been widely used in central and eastern U.S. as a bioindicator to detect phytotoxic levels of ambient ozone (Bennett and Jepson 1993; Bennett and Stolte 1985; Bennett et al. 2006; Bergweiler et al. 2008; Davis 2007b, 2011; Davis and Orendovici 2006; Myers et al. (2018); Smith et al. (2003); Yuska et al. 2003).

Table 1. Milkweed species' scientific and common names (adapted from USDA Plant Database Profiles, subspecies and synonyms not listed, *A. curassavica* not native to the U.S.); frequency of each species used by monarch butterflies in the eastern ("EAST") or western ("WEST") parts of North America (available online at https://www.fs.fed.us/wildflowers/pollinators/Monarch_Butterfly/habitat/milkweed_list.shtml. Accessed 31 October 2018; method used to estimate relative ozone-sensitivity and relevant reference(s) regarding ozone-sensitivity; common symptom(s); and overall ozone-sensitivity rating. In all columns, blank cells indicate no data.

[1]Scientific name	[2]Common name	[3]Used by monarchs in East or West	[4]Sensitivity evaluation method	[5]Evaluation reference	[6]Common symptom(s)	[7]Relative sensitivity rating
A. albicans S. Watson	Whitestem milkweed					
A. amplexicaulis Sm.	Clasping milkweed	EAST				
A. angustifolia Schweigg.	Arizona milkweed					
A. arenaria Torr.	Sand milkweed	EAST				
A. asperula (Decne.) Woodson	Spider milkweed	EAST	CSTR	Myers et al. 2018	STIP	SS
A. brachystephana Engelm. *ex* Torr.	Bract Milkweed					
A. californica Greene	California milkweed	WEST	FIELD	Temple 1999	NONE	R
A. cinerea Walter	Carolina milkweed					
A. connivens Baldw.	Largeflower milkweed					
A. cordifolia (Benth.) Jeps.	Heartleaf milkweed					
*A. cryptoceras*S. Watson	Pallid milkweed	WEST	FIELD	Davis 2017	NONE	R
A. curassavica L.	Tropical milkweed	EAST	CSTR	Hughes et al. 1990	STIP, DEFOL	VS
A. curtissii A. Gray	Curtiss' milkweed					
A. cutleri Woodson	Cutler's milkweed					
A. emoryi (Greene) Vail	Emory's milkweed					
A. engelmanniana Woodson	Engelmann's milkweed	EAST				

A. eriocarpa Benth.	Woolypod milkweed	WEST	FIELD	Allen et al. 2007	NONE	R
A. erosa Torr.	Desert milkweed	WEST				
A. exaltata L.	Tall milkweed		FIELD, OTC	Neufeld et al. 1992	CHL, STIP, DEFOL	VS
A. fascicularis Decne.	Mexican whorled milkweed	EAST	FIELD	Temple 1999	NONE	R
*A. feayi*Chapm. *ex* A. Gray	Florida milkweed					
A. glaucescens Kunth	Nodding milkweed	WEST				
*A. hallii*A. Gray	Hall's milkweed					
A. hirtella (Pennell) Woodson	Green milkweed	EAST	CSTR	Myers et al. 2018	NONE	R
A. humistrata Walter	Pinewoods milkweed					
A. hypoleuca(A. Gray) Woodson	Mahogany milkweed					
A. incarnata L.	Swamp milkweed	EAST	CSTR	Orendovici et al. 2003	STIP	VS
A. involucrata Engelm. *ex* Torr.	Dwarf milkweed					
A. labriformis M. E. Jones	Utah milkweed		FIELD	Davis 2017	NONE	R
A. lanceolata Walter	Fewflower milkweed					
A. lanuginosa Nutt.	Sidecluster milkweed					
A. latifolia (Torr.) Raf.	Broadleaf milkweed	EAST				
*A. lemmonii*A. Gray	Lemmon's milkweed					
A. linaria Cav.	Pineneedle milkweed	WEST				
A. linearis Scheele	Slim milkweed					
A. longifolia Michx.	Longleaf milkweed					
A. macrotis Torr.	Longhood milkweed					
*A. meadii*Torr. *ex* A. Gray	Mead's milkweed					
A. michauxii Decne.	Michaux's milkweed					
A. nivea L.	Caribbean milkweed					
A. nummularia Torr.	Tufted milkweed					
A. nyctaginifolia A. Gray	Mojave milkweed					
A. obovata Elliott	Pineland milkweed					
A. oenotheroides Schltdl. & Cham.	Zizotes milkweed	WEST				
A. ovalifolia Decne.	Oval-leaf milkweed	EAST	CSTR	Myers et al. 2018	STIP	SS
A. pedicellata Walter	Savannah milkweed					
A. perennis Walter	Aquatic milkweed					
A. physiocarpa(E. Mey.) Schitr.	Balloonplant					
A. prostrata W.H. Blackw.	Prostrate milkweed					
A. pumila (A. Gray) Vail	Plains milkweed	EAST				
A. purpurascens L.	Purple milkweed	EAST				
A. quadrifolia Jacq.	Fourleaf milkweed	EAST				
A. quinquedentate A. Gray	Slimpod milkweed					
A. rubra L.	Red milkweed					
A. rusbyi (Vail) Woodson	Rusby's milkweed					
A. scaposa Vail	Bear Mtn milkweed					

A. solanoana Woodson	Serpentine milkweed					
A. speciosa Torr.	Showy milkweed	EAST	CSTR	Myers et al. 2018	STIP	VS
A. sperryi Woodson	Sperry's milkweed					
*A. stenophylla*A. Gray	Slimleaf milkweed	EAST				
A. subulata Decne.	Rush milkweed	WEST				
A. subverticillata(A. Gray) Vail	Horsetail milkweed	WEST				
*A. sullivantii*Engelm. *ex* A. Gray	Prairie milkweed	EAST	CSTR	Myers et al. 2018	STIP	SS
A. syriaca L.	Common milkweed	EAST	OTC	Duchelle and Skelly 1981	STIP, DEFOL	VS
A. texana A. Heller	Texas milkweed					
A. tomentosa Elliott	Tuba milkweed					
A. tuberosa L.	Butterfly milkweed	EAST	CSTR	Myers et al. 2018	NONE	R
A. uncialis Greene	Wheel milkweed					
A. variegata L.	Redring milkweed	EAST				
A. verticillata L.	Whorled milkweed	EAST	CSTR	Myers et al. 2018	NONE	R
A. vestita Hook. &Arn.	Wooly milkweed	WEST				
A. viridiflora Raf.	Green comet milkweed	EAST				
A. viridis Walter	Green Antelopehorn milkweed	EAST	FIELD	Davis 2002	STIP	SS
A. viridula Chapm.	Southern milkweed					
*A. welshii*N.H. Holmgren & P.K. Holmgren	Welsh's milkweed					

[1]From USDA PLANTS Database Profiles

[2]Common names as frequently reported in the literature
[3]Frequency of milkweed species' use by monarchs. From: https://www.fs.fed.us/wildflowers/pollinators/Monarch_Butterfly/habitat/milkweed list.shtml (accessed 6 July 2017)
[4]Methodology used to rate sensitivity to: CSTR = Continuous Stirred Tank Reactor Chambers, FIELD = field surveys, OTC = Open-top ch ambient ozone
[5]Reference = authors' selected reference(s)
[6]Symptom: CHL = chlorosis, DEFOL = premature defoliation, STIP = adaxial leaf surface stipple, NONE = no symptom
[7]Relative sensitivity to ozone: VS = Very Sensitive, S = Sensitive, MS = Moderately Sensitive, SS = Slightly Sensitive, R = Resistant, Blank cell = unknown

Showman (1991) used common milkweed as a bioindicator species during ozone-injury surveys in southern Indiana and Ohiobut cautioned that the species exhibited little ozone injury in 1988, despite ambient ozone concentrations reaching the high level of 197 ppb. He concluded that assevere droughtin 1988 induced stomatal closure and reduced ozone uptake, limiting ozone-injury symptoms. Similarly, Miller et al. (1994) stated that the maximum ozone sensitivity of forest vegetation in California usually occurred in May, June, and July when stored soil moisture was adequate. Ozone-injury declined in August and September when soil moisture levels were depleted, in spite of high atmospheric ozone levels. Likewise, Kohut (2017) stated that historically low levels of precipitation and soil moisture on the Colorado Plateau may have constrained ozone uptake and limited ozone-induced symptoms on sensitive bioindicator species in some years. Smith et al. (2003, 2007) also reported that soil moisture stress could reduce ozone uptake and subsequent ozone-injury.

Regarding other milkweed species, Kline et al. (2009) compared the ozone-sensitivity of nine common milkweed selections from different locations in the Midwest and found considerable sensitivity, as well as considerable variation in response to ozone, among selections.

Kline et al. (2008) observed that native swamp milkweed was among the most sensitive of 28 plant selections. Orendovici (2002) and Orendovici et al. (2003)also rated swamp milkweed as the most sensitive of 40 plant species. They reported that main symptoms on swamp milkweed were chlorosis of older leaves followed by early leaf drop. Bolsinger et al. (1992) and Hughes et al. (1990) exposed the non-native (Mexican) tropical milkweed to ozone and rated the species as very sensitive to ozone based on the presence of leaf stipple and accelerated leaf senescence (premature defoliation) of stippled leaves. Neufeld et al. (1992) reported that tall milkweed was very sensitive to ozone, exhibiting foliar stipple during several years' exposures to ambient ozone in open-top field chambers. Chappelka et al. (1997, 2007), and Souza et al. (2006) also classified tall milkweed as sensitive to ambient ozone, based on stipple documented during field surveys. Souza et al. (2006) reported that ozone also induced premature defoliation on tall milkweed, and that most abscised milkweed leaves did not exhibit ozone-induced stipple prior to abscission. Davis (2002) rated green antelopehorn milkweed as slightly sensitive to ambient ozone, based on a trace of stipple observed during field surveys.

We recently exposed 11milkweed species to ozone in CSTR chambers within a greenhouse(Myers et al. 2018).Forcompleteness of this review, our recent ozone-sensitivity findings are discussed herein in some detail. The non-native tropical milkweed exhibited very high levels of ozone-induced stipple. Although indigenous to Mexico, tropical milkweed is widely planted in the U.S. in butterfly and pollinator gardens, and might serve as a bioindicator to detect phytotoxic levels of ambient ozone across wide areas of the U.S. However, the species also exhibited high levels of accelerated senescence (premature defoliation)in response to ozone, which was also reported by Hughes et al. (1990). Premature defoliation may compromise the usefulness of tropical milkweed as an ozone bioindicator, since stippled leaves may drop from the plant before they can be rated. In addition, non-stippled leaves might also drop, and thereafter cannot be not rated for ozone sensitivity. Further, it is difficult to correlate premature defoliation in the field with ambient ozone levels, since premature defoliation can be caused by various other environmental stress factors, such as drought or insect infestations. Nevertheless, the high level of ozone-induced leaf stipple and defoliation on tropical milkweed, reported in both in our study and that of Hughes et al. (1990) is significant, because levels of ozone in both studies were conducted near or below 70 ppb, the ozone concentration used in determining if violations of the current NAAQS have occurred. However, ozone concentrations and durations in these two studies differed from those prescribed in the NAAQS, making comparisons tenuous. In addition, the non-native tropical milkweed harbors a protozoan parasite (*Ophryocystiselektroschirrha*) that is infectious to monarch caterpillars that have fed on infected tropical milkweed leaves (Altizer et al. 2015, McLaughlin and Myers 1970, Satterfield et al. 2015). Because of this infectious parasite, it may be inadvisable to plant the non-native tropical milkweed outside of its native range when attempting to use the species as a bioindicator of phytotoxic ambient ozone, since healthy monarchs may contract the protozoan. It may be best to plant or manage only ozone-sensitive native milkweeds, including common milkweed, showy milkweed, and swamp milkweed, as bioindicators of phytotoxic levels of ambient ozone.

Prairie milkweed, showy milkweed, spider milkweed, and swamp milkweed, in addition to tropical milkweed, also developed characteristic ozone-induced stipple in our recent study(Myers et al. 2018). Summary ozone injury ratings (see calculations in Myers et al. 2018), in decreasing order of ozone-sensitivity, were tropical milkweed (0.624) > showy milkweed (0.497) > swamp milkweed (0.488) >common milkweed (0.208) > prairie milkweed (0.144) > spider milkweed (0.117) > butterfly milkweed (0.000) = green milkweed (0.000) = whorled milkweed (0.000). Based on these results, we consider tropical milkweed, showy milkweed, swamp milkweed, and common milkweed to be sensitive to ozone; prairie milkweed and spider milkweed to be slightly sensitive; and butterfly milkweed, green milkweed, and whorled milkweed to be tolerant of ozone.

Amember of the dogbane family, *Apocynumcannabinum* (Indian hemp dogbane), was reported to be sensitive to ozone, exhibiting foliar stipple after ozone exposure (Kline et al. 2009). A second *Apocynum* species, *A. androsaemifolium* (spreading dogbane), also has been classified as sensitive to ambient ozone in the field by several researchers (Davis 2007 a,b; Eckert et al. 1999; Kohut et al. 2000). Also, ozone may elicit adverse changes in sexual reproduction within spreading dogbane, in the absence of visible ozone-induced symptoms (Bergweiler and Manning 1999). Ozone has been reported to cause adverse effects on reproductive processes on other plant species (Leisner and Ainsworth 2012), but not for milkweed. If ozone is found to adversely affect the sexual reproduction of milkweed, then viable milkweed seed production and dissemination could be jeopardized.

Field biologists have rated some milkweed species as being tolerant to ambient ozone. Field tolerance of a milkweed species can be considered as a lack of ozone-induced stipple, when other ozone-sensitive plant species growing concurrently at the same location, exhibit ozone-induced stipple.

For example, Temple (1999) reported lack of stipple on California milkweed and Mexican whorled milkweed during field surveys within forests suffering from ozone injury.

Likewise, Allen et al. (2007) reported that wooly pod milkweed lacked symptoms when ozone-injury occurred on adjacent species. Also, Davis (2017) stated that pallid milkweed and Utah milkweed were tolerant during field surveys in Utah, when other plant species occasionally exhibited slight ozone injury. However, caution should be used when interpreting apparent ozone tolerance/resistance of milkweed species. As previously discussed, a species that is genetically sensitive to ozone might be mistakenly classified as "tolerant" due to drought-induced stomatal closure or low levels of ambient ozone, both of which might prevent development of diagnostic foliar symptoms. Conversely, a species that is genetically resistant to ozone is not likely to become injured when field environmental conditions are favorable for ozone uptake and development of visible ozone injury.

Summary ozone-sensitivity ratings for 75 U.S. milkweed species, plus tropical milkweed, are listed in Table 1. Although valuable, many of these ratings are subjective, often based on limited data at times and must be treated with caution. Nevertheless, the authors present this data in hopes that the ratings can be improved by subjecting various species of milkweeds to carefully controlled ozone exposurescombined with field surveys. Additional milkweed species within the genus *Asclepias* should be evaluated to determine if they could serve as valuable bioindicators to detect phytotoxic levels of ambient ozone, and to provide guidance for conducting further research dealing with ozone and milkweeds.

3. Relationship of Monarch Butterflies, Milkweeds, and Ozone

Eastern North American monarch butterflies are recognized worldwide for their annual north-to-south 5000 km (3000 mi), single-generation, north-to-south, migration that begins as far north as Canada. This amazing migrationends with a 4-month overwintering stop in Mexicoandthen proceeds with a multi-generational return flight the following spring (Agrawal 2017, Oberhauser and Solensky 2004, Oberhauser et al. 2015, Urquhart and Urquhart 1978).Ackery and Vane-Wright (1984)stated that spatiotemporal monarch population trends in North America during the migration maybe related to population trends of native milkweed host plants along the migration route. However, Agrawal (2017) related that eastern North America monarchs rely on milkweeds only during the breeding season, which occurs mainly during March to July in the eastern U.S. and Canada, rather than during migration. During the breeding season, adult females lay eggs only on milkweed plants and resultant caterpillars feed exclusively on milkweed leaves. During migration, adult monarchs need nectar as an energy source, which they get from a variety of plant species, including flowers of various milkweed species such as those presented in the USDA Plants Database Profiles (2017), which lists 22 *Asclepias* species frequently used by monarchs in eastern North America, and 10 *Asclepias* species frequently utilized in western North America (Table 1).

Regardless of cause,it is generally agreed that monarch populations have declined across the North America (Agrawal 2017, Malcom 2018, Thogmartin et al. 2017, USDA 2016). Although monarch declines may be caused by many interacting and/or cumulative factors (Agrawal 2017, Malcom 2018), the loss of common milkweed plants (due at least in part to agricultural herbicides) within the monarch's breeding range in the U.S. and Canada is considered an important factor in milkweed population declines (Borders and Lee-Mäder 2015, Pleasants and Oberhauser 2013).Milkweeds have long been recognized as common weed problems in agricultural fields along the monarch migration route in north-central U.S. and southern Canada (Bhowmik 1994). For decades, milkweed-infested fields along this route were treated with common herbicides, but milkweed species such as common milkweed persisted in populations that were great enough to benefit monarchs. With the advent of glyphosate herbicides approximately 20 years ago, followed by the subsequent release of glyphosate-tolerant corn and soybeans, milkweeds essentially have been eliminated from many agricultural fields along the migration route (Pleasants 2015). Borders and Lee-Mäder (2015) further stated "…loss of milkweed plants in North America is believed to be a major cause of recent monarch population declines." In addition to mortality, surviving milkweeds are subjected to chronic stresses from adverse environmental conditions, insect disorders, diseases, and other factors that cause premature defoliation (Hughes 1988, Pincebourde et al. 2017, Whitaker 1994). The authors of this review suggest that ozone-induced premature defoliation of milkweed should be recognized as a potential chronic stress to monarchs. In addition to causing direct defoliation, ambient ozone alters biochemical and physiological pathways within host plants that can affect plant relationships with leaf-chewing insects (Hughes 1988, Whitaker 1994).

Hughes et al. (1990) and Bolsinger et al. (1991, 1992) reported that monarch caterpillars had higher growth rates, leaf consumption rates, and developed faster on common milkweed and tropical milkweed leaves that had been exposed to ozone, as compared to non-exposed leaves.

Using tropical milkweed as the test plant, they related the feeding phenomena to biochemical changes in the milkweed leaves and suggested that enhanced feeding stimulation by monarchs may be the primary cause of the altered behavior and performance on ozone-exposed milkweeds. This phenomenon may relate back to the early observations of Howell (1974) who noted that polymers such as oxidized phenols formed in ozone-injured leaves might affect the nutritional value of ozone-injured foliage. However, this enhanced feeding stimulation ofozone-induced stippling of milkweed leaves is confounded by ozone-induced accelerated leaf senescence (premature defoliation). That is, ozone-induced stipple on milkweed leaves may initially stimulate monarch feeding, but in the presence of ozone, affected leaves (with or without stipple) may senesce and/or defoliate prematurely (Bolsinger et al. 1992, Hughes et al. 1990, Myers et al. 2018), which could negatively affect milkweed health and productivity. As reviewed earlier, premature defoliation in response to ozone has been reported for several milkweed species and may be a common response to ozone among sensitive milkweeds, whether the leaves are stippled or non-stippled. In addition, premature defoliation could remove deposited monarch eggs and resultant caterpillars from milkweed plants (Pleasants 2015). Such interactions between ozone-induced stipple, premature defoliation, and monarch health need additional research.

The annual north-to-south migration route ends in central Mexico. Many monarchs overwinter within the Monarch Butterfly Biosphere Reserve (MBBR), a World Heritage Site located ~120 km WNW of Mexico City, where monarch populations have been declining (Figure 2). The butterflies hibernate in high-elevation, cool, pine-fir forest stands dominated by sacred fir(*Abies religiosa*), but the threat from the air pollutant ozone does not end here. The greatest historic levels of ambient tropospheric ozone in North America have occurred in Mexico City. During the 1980s and 1990s, Mexico City was recognized as having one of the world's worst air pollution problems, both as a source and as a recipient (de Bauer and Hernández-Tejeda 2007). During decades of high ambient ozone, the former Mexican NAAQS ozone concentration of 110 ppb ozone was exceeded for 4–5 hours/day for more than 300 days in 1 year (year not given) (de Bauer and Hernández-Tejeda 2007).In 1990-1991, forests at the southwestern edge of Mexico City were exposed to 1-hour average ozone concentrations greater than 200 ppb for more than 10 days per year (Miller et al. 1994).The maximum ozone concentration recorded near Mexico City was 460 ppb, monitored on 19 March 1998 within the Desierto de los Leones National Park, located in the mountains on the SW edge of Mexico City (de Bauer and Hernández-Tejeda 2007).

Sacred fir is reported to be sensitive to ozone (de Bauer and Hernández-Tejeda 2007), and sacred fir stands, common at high elevations in this national park, have declined over the years, possibly related to past high ambient ozone levels (Alvarado et al. 1993). Although the sacred fir decline-syndrome, like many forest declines, may be compounded by secondary stresses (de Bauer and Hernández-Tejeda 2007), high concentrations of ambient ozone may have been the triggering agent in the decline (Alvarado et al. 1993). Fortunately, recent (1986–2014) monitoring has revealed reductions in ozone levels, likely related to the control of ozone-forming precursors implemented in Mexico City (Barrett and Raga 2016).

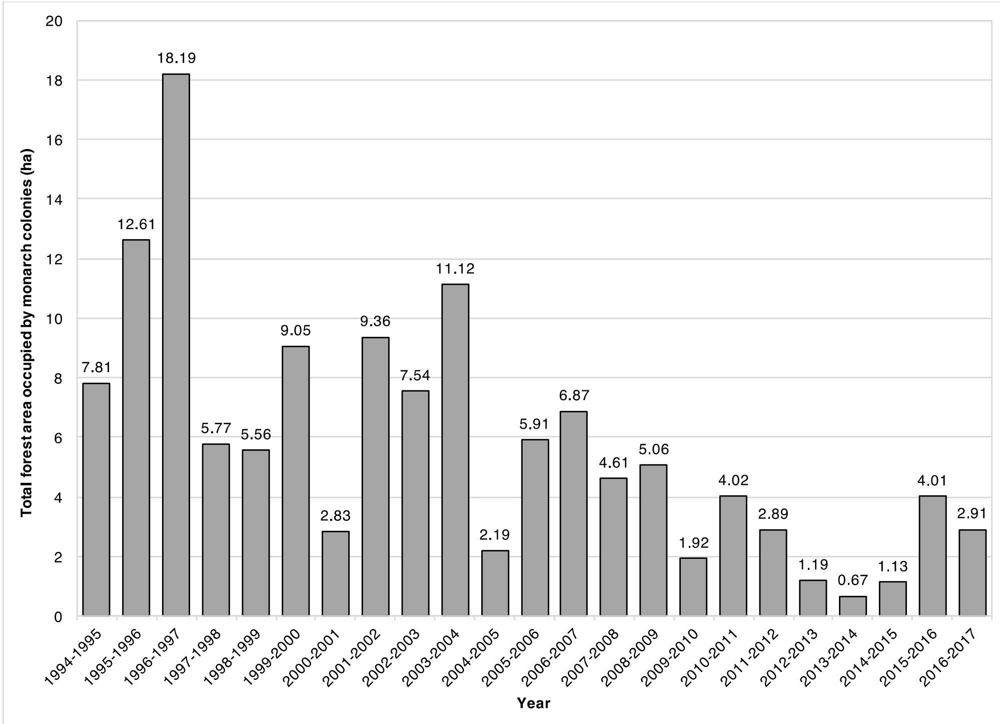


Figure 2. Total forest area (ha) occupied by monarch butterfly colonies at overwintering sites in Mexico during the time period from winter 1994/1995 to winter 2016/2017. Original data from 1994–2003 (except for 2000–2001) were collected by personnel of the Monarch Butterfly Biosphere Reserve (MBBR), National Commission of Protected Natural Areas (CONANP) in Mexico. The 2000–2001 data was reported by Garcia-Serrano et al. (2004). Data from 2004–2016 were collected by the WWF-Tecel Alliance, in coordination with the Directorate of the MBBR. Data converted from monarch butterfly numbers to density (Thogmartin et al. 2017).

If ambient ozone levels are involved in the sacred fir decline, then reduction of ozone-forming precursors may slow or mitigate the decline. elatedly, the MBBR where the monarchs overwinter on sacred fir at high elevations, is located west of the national park and slightly upwind from Mexico City. If high levels of ambient ozone did impinge on the reserve, the stands of the ozone-sensitive sacred fir might decline further, endangering hibernating monarch populations. However, the authors found no reports that sacredfirs in the MBBR are currently impacted by ozone, which is endangering hibernating monarchs. Unfortunately, ambient ozone monitoring is not conducted in or near the MBBR. However, the Real-time Air Quality Index Visual Map

(available online at http://aqicn.org/map/world/#@g/19.6511/-99.9179/9z accessed 12 July 2017) reveals a cluster of five air pollution monitors within the city of Toluca,located~75 km SE of the MBBR and ~37 km west of the national park. These monitors indicate that the Air Quality Index (which includes ozone, but ozone values are not listed separately), is less in Toluca than at the edge of Mexico City, suggesting that Toluca may be somewhat upwind of Mexico City and not impacted by high levels of ozone. However, interpretation of modeling/prediction of ambient ozone concentrations in or near the MBBR is beyond the scope of this review.

Caution must be utilized when drawing conclusions relating ozone to monarch population declines from the specific examples above, since the examples are simplifications of complex interactions among the host plant, insect, environment, and pattern of ozone exposure (Whittaker 1994).Hughes et al. (1990) summarized the state of ozone-milkweed-monarch research in 1990 by stating, "…there is now a need to move to field studies…that will permit assessment under more natural conditions of plant growth and pollutant exposure. Such field studies will also permit better exploration of the importance of accelerated leaf senescence to monarch survival as well as the effect of ozone on nectar composition, which is critical to pollen germination, and hence to seed production by the milkweed."

Obviously, nearly 30 years later, additional scientific research is still needed, perhaps now more than ever, in this complicated, interwoven, tripartite ecology of ambient ozone, milkweeds, and monarch butterflies. We hope this review will stimulate such research and provide a framework for further studies.

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