Title

Towards a wild fermentation ecology: alcohol within floral nectar and the frugivorous diet of chimpanzees

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Towards a wild fermentation ecology:
alcohol within floral nectar and the frugivorous diet of chimpanzees

By

Aleksey Maro

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Integrative Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Robert Dudley, Chair

Professor Noah K. Whiteman

Professor Christine A. Hastorf

Spring 2026

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Abstract

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Professor Robert Dudley, Chair

Alcohol, specifically ethanol, is estimated to have been consumed by 46% of the world's population above the age of 15 in 2019 [**1**]. Human societies have been intentionally brewing alcohol for consumption for at least 9,000 years [**2**], likely longer [**3**]. Why are humans so attracted to ethanol consumption? Is it merely the result of a happy biochemical accident? Or could there be an evolutionary explanation? The "drunken monkey" hypothesis points out that the ancestors of humans were frugivorous for tens of millions of years and predicts that the fruits they ate underwent microbial fermentation resulting in the accumulation of ethanol [**4**]. Olfaction of fruit ethanol by foraging animals may facilitate the localization of fruit crops and indicate the suitability of individual fruits for consumption. The ingestion of fruit ethanol may itself provide beneficial services such as appetite stimulation. Physiological tolerance of increasingly ethanolic fruits by primates and other animals may therefore have been adaptive over evolutionary time. The "drunken monkey" hypothesis thus receives its name from the prediction that an evolutionary bottleneck resulted in a period when intoxication was a dietary tradeoff for survival among our as yet ethanol-naive ancestors. Such a mismatch can be seen among the contemporary well-studied alcohol drinking habits of the vervet monkeys on the island of St. Kitt's in the Caribbean, which are exposed to highly concentrated cocktails left by tourists, and which are frequently observed to be visibly intoxicated [**5**]. Thus, the "drunken monkey" hypothesis suggests that the contemporary human propensity for alcohol is the result of our evolutionary heritage of chronic fruit consumption.

This dissertation began with the aim of collecting basic empirical fruit alcohol data to test the "drunken monkey" hypothesis, by estimating whether the fruits consumed by wild chimpanzees today contain ethanol in sufficient concentrations to amount to a physiologically relevant dosage. I was then also able to collect direct physiological evidence in support of this hypothesis, by measuring a liver metabolite of ethanol (ethyl glucuronide) in the urine of chimpanzees. Chimpanzees were chosen as the study subject for the first two chapters because

they, along with bonobos, are the closest living relatives to humans, and thus their ethanol-related physiology is the most likely to correspond to that of our ancestors who lived a similar forest-dwelling lifestyle. The third chapter consists largely of a broad multispecies survey of floral nectar ethanol in the university's botanical garden. I used published studies to determine the pollinator associated with each species of flower in its native range. I then found studies the on the field metabolic rate of some of those pollinators, and estimated the ethanol dosage that they would consume on an average day of foraging. This third chapter is what broadens the scope of the dissertation beyond testing the "drunken monkey" hypothesis towards the search of a broader wild fermentation ecology.

Chapter 1 shows that chimpanzee-consumed fruits at two field sites contained ~0.3% ethanol, and that the average daily rate of fruit consumption of wild chimpanzees equates to ~14 grams of pure ethanol, or the equivalent to 1–2 standard drinks per day by human standards. Three distinct methods of ethanol assay were used in this chapter, two that measured the concentration of ethanol in the headspace of fruit pulp, and one method that detected ethanol in centrifuged fruit juice by changing its color with chemical reagents. This chapter was published in the journal *Science Advances* [**6**].

Chapter 2 shows that the majority of chimpanzees whose urine was sampled passed a concentration threshold of 500 ng/mL ethyl glucuronide, indicating a daily consumption of at least 1–2 standard drinks as evidenced by human studies. The method consists of applying a few drops of chimpanzee urine to a lateral flow dipstick immunoassay similar to a pregnancy test, offering a cheap field-portable method for estimating the relative exposure of wild animals to ethanol in lieu of a breathalyzer. This method is well suited for dietary studies because it reflects alcohol consumed over a period of 24–48 hours as the metabolite accumulates in the bladder. This chapter was published in the journal *Biology Letters* [**7**].

Chapter 3 shows that ethanol can be found across many species of flowers and in a high percentage of samples. The ethanol concentrations appear low, on the order of 0.02%, but given the large daily volume of nectar that pollinators such as hummingbirds consume, as much as 192% of their body mass, this adds up to a physiological dosage equivalent to 1–2 standard drinks per day by human standards. The method used was an exceptionally sensitive enzyme-based assay kit. This chapter was published in the journal *Royal Society Open Science* [**8**].

This dissertation offers an empirical foundation for the continuing study of wild fermentation ecology. There is no longer any ambiguity that fruit- and nectar-feeding animals are chronically exposed to low concentrations of ethanol. It remains to be shown whether or not seed dispersers and pollinators actively prefer ethanol or are simply unable to avoid it. Future studies should match fruit/nectar ethanol data with more direct observations of the microbial community and its effects on animal foraging decisions. While the microbial ecology of floral nectar has been relatively well documented, the microbial community ecology of fruit pulp remains virtually unstudied in the wild. The next logical step is to describe the microbial community

assembly underpinning wild fruit and nectar fermentation, which is crucial for a complete understanding of the dietary ecology of fruit and nectar eaters, including that of our own species.

The raw data for each chapter can be found in the supplementary materials of its respective open access publication [**6**–**8**].

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1. World Health Organization. 2024 *Global status report on alcohol and health and treatment of substance use disorders*. Geneva, Switzerland: World Health Organization.
2. Liu L, Zhang J, Li J, He Y, Gao Z, Jiang L. 2024 Identification of 10,000-year-old rice beer at Shangshan in the Lower Yangzi River valley of China. *Proc. Nat. Acad. Sci.* **121**, e2412274121. (doi:10.1073/pnas.2412274121)
3. Liu L, Wang J, Rosenberg D, Zhao H, Lengyel G, Nadel D. 2018 Fermented beverage and food storage in 13,000 y-old stone mortars at Raqefet Cave, Israel: Investigating Natufian ritual feasting. *J. Archaeol. Sci. Rep.* **21**, 783–793. (doi:10.1016/j.jasrep.2018.08.008)
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6. Maro A, Sandel AA, Blaiore BZA, Wittig RM, Mitani JC, Dudley R. 2025 Ethanol ingestion via frugivory in wild chimpanzees. *Sci. Adv.* **11**, eadw1665. (doi:10.1126/sciadv.adw1665)
7. Maro A, Byrne LC, Namaganda S, Dudley R. 2026 Urinary concentrations of a direct ethanol metabolite indicate substantial ingestion of fermenting fruit by chimpanzees. *Biol. Lett.* **22**, 20250740. (doi:10.1098/rsbl.2025.0740)
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List of Figures and Tables

Acknowledgments

Many acknowledgments may be found in the peer reviewed publication corresponding to each chapter (see Abstract above). Thank you to Noah Whiteman and Christine Hastorf for serving on my dissertation committee. I am also grateful for the mentorship of my qualifying exam committee Leslea Hlusko (chair), Noah Whiteman, Ellen Simms, and Tom Bruns.

Curriculum Vitae

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Education

2017–2026: Ph.D. in Integrative Biology, University of California, Berkeley
Advisor: Robert Dudley
Dissertation title: *Towards a wild fermentation ecology: alcohol within floral nectar and the frugivorous diet of chimpanzees*

2013–2015: B.A. in Integrative Biology, University of California, Berkeley – *high honors*
Honor's thesis title: *Biogeography of ungulate-visited salt licks in North America*

Professional affiliations

2017–present: American Association of Biological Anthropologists (American Association of Physical Anthropologists prior to 2019)
2018–present: Society for Integrative and Comparative Biology (SICB)
2019–present: Museum of Vertebrate Zoology at the University of California, Berkeley

Publications

Maro A, Corl A, Bowie R, McGuire J, Dudley R. (2026). Low-level ethanol is widespread within floral nectar. *Royal Society Open Science* 13, 250847.

Maro A, Byrne LC, Namaganda S, Dudley R. (2026). Urinary concentrations of a direct ethanol metabolite indicate substantial ingestion of fermenting fruit by chimpanzees. *Biology Letters* 22, 20250740.

Maro A, Sandel AA, Blaiore BZA, Wittig RM, Mitani JC, Dudley R. (2025). Ethanol ingestion via frugivory in wild chimpanzees. *Science Advances* 11, eadw1665.

Boucher RD, Wittig RM, Lemoine SR, **Maro A**, Wang X, Koch PL, Oelze VM. (2024). Strontium isotopes track female dispersal in Taï chimpanzees. *American Journal of Biological Anthropology* 184, e24981.

Choi J, Lee L, **Maro A**, Corl A, McGuire JA, Bowie RC, Dudley R. (2023). Hummingbird ingestion of low-concentration ethanol within artificial nectar. *Royal Society Open Science* 10**,** 230306

Campbell CJ, **Maro A**, Weaver V, Dudley R. (2022). Dietary ethanol ingestion by freeranging spider monkeys (Ateles geoffroyi). *Royal Society Open Science* 9, 211729.

Maro A, Dudley R. (2022). Non-random distribution of ungulate salt licks relative to distance from North American oceanic margins. *Journal of Biogeography* 49, 254–260.

Dudley R, **Maro A**. (2021). Human Evolution and Dietary Ethanol. *Nutrients* 13, 2419.

Manuscripts in preparation

Maro A, Sandel AA, Blaiore BZA, Wittig RM, Mitani JC, Dudley R. Variation in ethanol concentrations and physical attributes of wild chimpanzee-consumed fruits.

Research experience

2017–present: Graduate Researcher

Department of Integrative Biology, University of California, Berkeley

Summer 2025: Series of experiments at Ngogo, Kibale National Park, Uganda assaying fermentation of chimpanzee-consumed fruits, culturable microbes using fruit agar, and assessment of ethanol metabolites in urine, (published in *Biology Letters*).

Fall 2024: Comparative analysis of alcohol metabolic gene regions within hummingbirds and between avian lineages (in collaboration with Ammon Corl) .

Fall 2023–Summer 2023: Preliminary work estimating selection over time for alcohol related mutations in Europeans using modern and ancient DNA (in collaboration with Rasmus Nielsen and Andrew Vaughn).

Spring 2022–2023: Assays of ethanol within floral nectar at the UC Botanical Garden (published in *Royal Society Open Science*).

Summer 2021: Field season in Taï National Park, Côte d'Ivoire assaying ethanol content of the primary fruits consumed by chimpanzees (published in *Science Advances*).

Fall 2020: Pilot study at the Santa Rita Experimental Range assaying ethanol content of prickly pear cactus fruit (*Opuntia* spp.) .

Spring 2020: Built a phylogeny of all available (NCBI) *alcohol dehydrogenase* (*ADH*) gene family protein coding sequences to evaluate *ADH* evolution across vertebrates.

Fall 2019: Field season in Ngogo, Kibale National Park, Uganda refining methods for measuring ethanol content within chimpanzee-consumed fruit, and surveying ethanol concentrations in ambient air around fruit trees (published in *Science Advances*).

Spring 2019: Evaluated *ADH* coding sequences of 70 chimpanzees and bonobos against known human *ADH* polymorphisms.

Summer 2019: Pilot study analyzing ethanol concentrations of wild prickly pear cactus fruit (*Opuntia chlorotica*) near Joshua Tree National Park, CA.

Summer 2018: Field season in Ngogo, Kibale National Park, Uganda measuring ethanol content of chimpanzee-consumed fruit (published in *Science Advances*).

2015 (Spring & Fall): Undergraduate research

Department of Integrative Biology, University of California, Berkeley

Honor's thesis: Analysis of geospatial and altitudinal data for mineral licks in North America (published in the *Journal of Biogeography*)

Experiments testing Anna's Hummingbird sensitivity to induced static charge on nectar feeders, unpublished negative results (collaboration with Victor Ortega)

2014 (Spring) Undergraduate Research Apprenticeship Program

College of Natural Resources, University of California at Berkeley

Apprenticeship with: Suding Lab, Lauren Hallett (Hallett Lab)

Teaching experience

2017–present: Graduate Student Instructor (13 semesters + 1 summer)

Spring 2026: Evolutionary Medicine discussion – IB169

Fall 2025: Practical Genomics lab – IB134L

Spring 2025: Physiology lab – IB132L & Evolutionary Medicine discussion – IB169

Summer 2024: Infectious Disease Dynamics – IB114

Spring 2024: Evolutionary Medicine discussion – IB169

Fall 2023: Human Anatomy lecture & lab (head GSI) – IB131/131L

Spring 2021 – 2023: Physiology lab (head GSI; 3 semesters) – IB132L
Spring 2020: Physiology lab – IB132L
Spring 2019: Ornithology lab/field – IB174LF
Fall 2017 – Fall 2018: General Biology lab (3 semesters) – Bio1B 2016–2017

2016–2017: Museum Educator at the Lawrence Hall of Science, University of California, Berkeley. Designed curriculum and taught STEM-oriented camps and workshops for various ages.

Conference presentations

2026 American Association of Biological Anthropologists (AABA) in Denver, CO – Poster presentation (session chair): *Substantial fruit ethanol ingestion by wild chimpanzees suggested by physiologically high urinary concentrations of ethyl glucuronide*

2024 Society for Integrative and Comparative Biology (SICB) in Seattle, WA – Oral presentation (best student talk finalist): *A survey of ethanol concentrations within floral nectars at a Mediterranean climate botanical garden*

2023 American Association of Biological Anthropologists (AABA) in Reno, NV – Oral presentation: *A comparison of ethanol concentrations in chimpanzee consumed fruits at lowland and mid-altitude sites*

2023 Society for Integrative and Comparative Biology (SICB) in Austin, TX – Oral presentation (session chair) and virtual platform (SICB+): *Biogeographic distribution of salt licks relative to oceanic margins in North America*

2023 Society for Integrative and Comparative Biology (SICB) in Austin, TX – Poster presentation and virtual platform (SICB+): *Field portable methods for the quantification of fruit ethanol concentrations*

2021 American Association of Biological Anthropologists (AABA) virtual throwback program oral video presentation (same title as 2020); winner of Outstanding Student Presentation Award ($500).

2020 American Association of Physical Anthropologists (AAPA) in Los Angeles, CA (virtual oral slide recording due to COVID-19) *Dietary ethanol in the main food (*Ficus mucuso*) of chimpanzees* (Pan troglodytes*) in a tropical rain forest*

2019 Society for Integrative and Comparative Biology (SICB) in Tampa, FL – Oral presentation: *Ethanol concentrations within primate-consumed fruit in a tropical rainforest*

Lectures and seminars

2026 UC Santa Cruz; Guest lecturer, Primate Behavior and Ecology (ANTH106) – *Chimpanzee dietary ecology and the evolutionary origins of human alcohol consumption*

2026 Popping the Science Bubble at the Berkeley Public Library – *Wild fermentation, the chimpanzee diet, and the origins of alcohol production*

2026 Nerd Nite at the Rickshaw Stop in SF – *Wild fermentation, the chimpanzee diet, and the origins of alcohol production*

2026 Ph.D. finishing talk at the MVZ Grinnell Library – *Towards a wild fermentation ecology: alcohol within floral nectar and the frugivorous diet of chimpanzees*

2026 UC Berkeley; Museum of Vertebrate Zoology Annual Symposium – *In pursuit of an evolutionary hangover*

2025 UC Berkeley; Guest lecturer, Evolutionary Medicine (IB169) – *The 'Drunken Monkey' hypothesis, wild fermentation ecology, and the origins of alcohol consumption*

2025 UC Berkeley; Museum of Vertebrate Zoology Annual Symposium – *Grad to postdoc activities*
2024 UC Berkeley; Museum of Vertebrate Zoology Annual Symposium – *Using ancient DNA to investigate alcohol-related genomic mutations in Europeans*
2023 University of Bordeaux 'Tipping Points in Human Evolution' invited guest speaker – *Towards a wild fermentation ecology: ethanol concentrations within chimpanzee consumed fruit and within floral nectar*
2023 UC Berkeley; Museum of Vertebrate Zoology Annual Symposium – *Ethanol concentrations within flower nectar*
2022 UC Berkeley; Department of Integrative Biology Annual Symposium – *(Mis)adventures in field primatology*
2022 UC Berkeley; Museum of Vertebrate Zoology Annual Symposium – *Fruit ethanol concentrations within chimpanzee-consumed fruit*; winner of the Jim Patton Field Survival Award
2021 Centre Suisse de Recherches Scientifiques, Abidjan, Côte d'Iviore; post-field research presentation – *Ethanol concentrations within fruit species consumed by chimpanzees in Taï National Park, Côte d'Ivoire*
2021 UC Berkeley; Seminar in Physiological Energetics and Biomechanics – *Chimpanzee foraging behavior and dietary ethanol intake*
2020 UC Santa Cruz; Anthropology Department seminar, invited speaker – *Ethanol within chimpanzee-consumed fruit: presence, preference, and significance*
2020 UC Berkeley; Museum of Vertebrate Zoology Annual Symposium – *Ethanol within chimpanzee-consumed fruit: presence, preference, and significance*
2019 Max Plank Institute, Leipzig, Germany; Primatology Department seminar, field research proposal presentation – *The drunken monkey: ethanol concentrations within chimpanzee-consumed fruit in a tropical rainforest*
2018 UC Berkeley; Seminar in Physiological Energetics and Biomechanics – *Salt deprivation in herbivores, a non-random spatial distribution of salt licks in North America*

Professional development
2025 Stanford's PRISM program for prospective postdocs
2024 Certificate of Teaching and Learning in Higher Education
2022–2023 Outstanding Graduate Student Instructor Award, Department of Integrative Biology
2021 Graduate Remote Instruction Innovation Fellows Program Certificate in Remote Instruction

Peer review
2023 *Ecology and Evolution*
2023 *Proceedings of the Royal Society B* co-reviewer
2023 *Oikos*
2022 *Journal of Animal Ecology*
2022 *PLoS ONE*
2022 National Science Centre of Poland

Awards/grants
2025 Department of Integrative Biology Summer Research Award ($4000)
2024 Department of Integrative Biology Summer Research Award ($5000)

2023 Department of Integrative Biology Dissertation Completion Award ($2500)
2022 Outstanding Graduate Student Instructor Award, Department of Integrative Biology ($250)
2022 Museum of Vertebrate Zoology Annual Symposium Jim Patton Field Survival Award
2022 Department of Integrative Biology Summer Research Award ($2383)
2021 AABA Outstanding Student Presentation Award ($500)
2021 Department of Integrative Biology Summer Research Award ($1670)
2021 Graduate Remote Instruction Innovation Fellows Program ($2000)
2020 Berkeley Sigma Xi Grants In Aid of Research (GIAR) Award ($500)
2020 Hamilton Company Syringe Grant Program Award ($1000)
2020 Department of Integrative Biology Summer Research Award ($1300)
2020 Museum of Vertebrate Zoology Karl Kroford Research Funds ($1200)
2019 Pollitzer Travel Award to the AABA Conference in Cleveland, OH ($500)
2019 Department of Integrative Biology Summer Research Award ($700)
2019 Museum of Vertebrate Zoology Karl Kroford Research Funds ($2000)
2015 Gilman International Scholarship to study in the Czech Republic ($1800)

Media coverage

2026 representative media coverage of **Maro et al., 2026** *Low-level ethanol is widespread within floral nectar*:

"Колібрі виявилися затятими алкоголіками, але їм це не заважає, - вчені", *Unian* (4 April 2026)
"Selbst Bienen und Kolibris trinken Alkohol – er steckt im Nektar", *Merkur* (3 April 2026)
"Ces animaux qui boivent (aussi) de l'alcool", *Sciences et Avenir* (30 March 2026)
"Por qué los colibríes no se embriagan pese a consumir 'alcohol' todos los días", *La Nación* (28 March 2026)
"Пьют как люди: ученые подсчитали ежедневную дозу алкоголя у колибри и пчел", *Hayka* (28 March 2026)
"Hummingbirds Drink a Ton of Alcoholic Nectar, But Do They Get Drunk?", *Vice* (27 March 2026)
"Колибри уличили в регулярном употреблении алкоголя", *naked-science.ru*, (26 March 2026)
"Por que beija-flores não ficam bêbados mesmo tomando álcool todo dia", *Galileu* (26 March 2026)
"鳥や蜂が花蜜中のアルコールを摂取することを確認（Birds do it, bees do it … sip alcohol, that is)", *Tiisys.com* (25 March 2026)
"Koolibrid ja mesilased joovad iga päev alkoholi", *ERR News* (25 March 2026)
"Beija-flores consomem álcool durante polinização, aponta novo estudo", *Metrópoles* (25 March 2026)
"Hummingbirds drink equivalent of a pint of beer a day", *The Telegraph* (25 March 2026)

2026 representative media coverage of **Maro et al., 2026** *Urinary concentrations of a direct ethanol metabolite indicate substantial ingestion of fermenting fruit by chimpanzees*:

"Humans' pull toward alcohol may have ancient origins (according to chimp pee)", *NPR* (Mar. 3, 2026)
"A csimpánzok tényleg iszákosak", *index.hu* (Mar. 1, 2026)
"Les chercheurs confirment une consommation d'alcool chez les chimpanzés grâce à l'analyse de leurs urines", *Science & Vie* (28 Feb. 2026)
"Wild Chimps Get Drunk on a Daily Basis, Scientists Find", *Vice* (27, Feb. 2026)
"Schimpansen und Alkohol: Warum wir Alkohol lieben", *Die Zeit* (26 Feb. 2026)
"Gli scimpanzé selvatici dell'Africa positivi all'alcol-test", *Quotidiano Nazionale* (25 Feb. 2026)
"Обнаружилось, что дикие шимпанзе живут в состоянии легкого подпития", *nauka.ru* (25 Feb. 2026)
"Wild chimpanzees love to eat boozy fruit", *Smithsonian Magazine* (25 Feb. 2026)
"Wild chimpanzees would 'fail sobriety tests'", *The Times* (London) (25 Feb. 2026)
"Soviel alcohol konsumieren wildlebende Schimpansen", *wissenschaft.de* (25 Feb. 2026)
"黑猩猩血液酒精含量检测呈阳性: 每天大约喝一到", *mandarinian.news* (25 Feb. 2026)
"Wild chimpanzees may be consuming two alcoholic drinks a day", *Discover Magazine* (25 Feb. 2026)
"Chimpanzee pee reveals how our primate cousins are getting drunk on fermented fruit", *Scientific American* (25 Feb. 2026)

"야생침팬지 소변 샘플 20개 검사…17개서 알코올 대사물 검출", *Yohap News* (25 Feb. 2026)
"Chimpanzees love alcohol and their pee proves it", *Popular Science* (24 Feb. 2026)

2026 ***The Wild Bits*** (podcast), Feb 22. I discuss my PhD research during an hour-long interview.

2025 Featured in the documentary series ***Tiere im Rausch* (*Wild on a High*)** conducting research on chimpanzee-consumed fruits at Ngogo in Uganda.

2025 representative media coverage of **Maro et al., 2025** Ethanol ingestion via frugivory in wild chimpanzees:

"Blame our love of booze on our primate ancestors", *The Wall Street Journal* (29 Nov. 2025)
"Chimps imbibe two cocktails' worth of ethanol from ripe fruits", *The Hindu* (22 Oct. 2025)
"Μελέτη / Οι χιμπατζήδες καταναλώνουν τακτικά αλκοόλ", *tvxs.gr* (13 Oct. 2025)
"La ciencia confirma la 'teoría del mono borracho'", *Las Provincias* (30 Sept. 2025)
"Wild chimps consume the equivalent of two glasses of wine a day", *Popular Science* (29 Sept. 2025)
"Schimpanser får i sig "två drinkar" om dagen", *Göteborgs-Posten* (28 Sept. 2025)
"Chimpanzees' taste for ripe fruit is equivalent to two drinks a day", Podcast *Quirks and Quarks* (27 Sept, 2025)
"How wild chimpanzees get their daily alcohol buzz from fermented fruit", *Times of India* (25 Sept. 2025)
"UC Berkeley drunken monkey hypothesis", Live on *NBC Bay Area* (23 Sept. 2025)
"Chimps consume the equivalent of 2.5 alcoholic drinks per day by eating fermented fruit, study finds", *Smithsonian magazine* (23 Sept. 2025)
"Wild chimps eating fermenting fruit get a surprising slug of alcohol", Live on *NewsNation Now* (19 Sept, 2025)
"Betrunkener-Affe-Hypothese – Was unser Feierabendbier mit den Vorlieben der Schimpansen zu tun hat", *Der Spiegel* (19 Sept. 2025)
"Chimpanzees may consume equivalent of 1 alcoholic drink a day from fermented fruit, study finds", *CNN Science* (18 Sept. 2025)
"Les chimpanzés sauvages avalent l'équivalent de deux verres de vin par jour", *Le Figaro* (18 Sept. 2025)
"Исследование: обезьяны употребляют эквивалент бутылки пива в день", *mk.ru* (18 Sept. 2025)
"Schimpansen konsumieren täglich Alkohol", *Österreicher Rundfunk* (18 Sept. 2025)
"Wild chimps eating fermenting fruit get a surprising slug of alcohol", *reuters.com* (18 Sept. 2025)
"Schimpansen konsumieren jeden Tag Alkohol", *Stern* (18 Sept. 2025)
"Yes, wild chimps consume alcohol. Here's how much – and what that tells us about humans", *San Francisco Chronicle* (17 Sept. 2025)
"Chimps consume equivalent of a pint of lager every day", *The Times (London)* (17 Sept. 2025)
"Wild chimpanzees consume 2 cocktails worth of alcohol a day by feasting on boozy fruit", *nypost.com* (17 Sept. 2025)
"Los chimpancés toman el alcohol equivalente a dos o tres cañas de cerveza al día", *El País* (17 Sept. 2025)
"Chimps consume equivalent of a beer a day in alcohol from fermented fruit", *The Guardian* (17 Sept. 2025)
"Wild chimpanzees may get mildly intoxicated from alcoholic fruit", *NewScientist* (17 Sept. 2025)
"Des chimpanzés accros à l'alcool? Notre penchant pour l'apéro viendrait de là", *Le Parisien* (17 Sept. 2025)
"Chimps drinking a lager a day in ripe fruit, study finds", *BBC* (17 Sept. 2025)

2022 Interviewed in ***Taste: The Flavor of Life***, a documentary series about evolution and the sense of taste on Curiosity Stream.

Chapter 1

Ethanol ingestion via frugivory in wild chimpanzees

1.0 Abstract

Human attraction to alcohol may derive from an evolutionary association between ethanol and fruits consumed by animals in nature. Fermentative yeasts are widespread in the terrestrial biosphere, and simple carbohydrates underpinning ethanol production are commonplace within fruits. We determined ethanol concentrations within fruits representing a substantial portion of the diet of our closest living relatives, the chimpanzees. Ripe fruit pulp from twenty angiosperm species in Côte d'Ivoire and Uganda contained an average value of 0.31 (± 0.21 standard deviation) and 0.32% (± 0.20) ethanol (weight/weight), respectively, as scaled by annual chimpanzee feeding time per species at each site. Chimpanzees typically eat ~4.5 kg of fruit per day, corresponding to an estimated ethanol ingestion of 14 g (±9), or the equivalent of 1.4 (±0.9) standard drinks by international standards. These findings are consistent with the hypothesis that ethanol is widespread within tropical fruits, and that modern predisposition to alcohol consumption derives from ancestral exposure to this psychoactive substance among frugivorous primates.

1.1 Introduction

Habitual use of alcohol by modern humans is common around the world. The earliest archaeological evidence for controlled fermentation dates to 9,000 – 13,000 years ago in China and in the Middle East [**1**,**2**]. Intentionally directed fermentation, as a form of predigestion for enhanced caloric extraction, is likely much older [**3**,**4**]. Nevertheless, humanity's attraction to alcohol, both ancient and present, may have deeper evolutionary roots given exposure of our frugivorous ancestors to ethanolic fermentation [**5**]. Ethanol, used here synonymously with alcohol, has been hypothesized to be commonplace within the primarily frugivorous diet of our Afrotropical hominid ancestors, resulting in evolution of adaptive physiological responses to its dietary consumption, i.e., the "drunken monkey" hypothesis [**6**]. For example, ethanol may function as an olfactory and gustatory indicator of caloric status associated with consumption of ripe fruit, a sensory modality that offers supplemental benefits to any animal that utilizes sugars as part of its primary or secondary diet [**7**]. To begin to test such hypotheses, which call for a broader understanding of the evolutionary ecology of ethanol [**8**], describing exposure of chimpanzees (*Pan troglodytes*) to dietary ethanol in their natural habitat is of particular interest. Chimpanzees are one of our two closest living relatives and, along with nearly all extant apes, chronically consume large volumes of ripe fruit. This frugivorous diet is thought to be similar to the diet of our last common ancestor with chimpanzees [**9**,**10**], although it has been suggested that the chimpanzee lineage has continued to evolve toward higher ripe fruit specialization [**11**]. Characterizing ethanol concentrations of these fruits, and estimating the daily ingested volume of ethanol, may accordingly provide insights into its availability to early hominins.

Ethanol is the primary product of anaerobic catabolism of sugars by microbes, typically yeasts. Ethanol production in the yeast family Saccharomycetaceae, to which *Saccharomyces cerevisiae* or brewer's yeast belongs, has been under natural selection for over 100 million years [**12**,**13**]. In these Crabtree-positive yeasts, ethanol is produced via fermentation even in the presence of oxygen, which provides yeast only one-ninth of the energy yield that would otherwise come from oxidative metabolism [**14**]. This trait may have evolved as part of a 'make–accumulate–consume' strategy, wherein accumulated ethanol acts as a microbicide to initially enhance competition in sugary substrates, followed by reproductive growth as yeasts metabolize the ethanol [**15**]. A sugar-rich and progressively more anoxic environment thus facilitates ethanolic fermentation, whereby both facultative and obligate anaerobes compete with one another. Adaptations facilitating these strategies arose concurrently with the worldwide radiation of angiosperms >100 million years ago (Ma) [**16**], and thus coincided with the evolution of fleshy fruits and associated seed dispersal by frugivores [**17**]. Such adaptations may have evolved as part of a larger mutualistic web [**8**], wherein yeasts receive calories and substrate from the angiosperm in exchange for providing a suite of ecological services to seed dispersers. By biochemically modifying the fruit niche and producing ethanol, yeasts offer a potential long-range signal of calories via ethanol plumes [**7**], an indication of individual fruit quality via olfaction [**18**], and stimulation of appetite during ingestion [**19**]. Fermentative yeasts may thus play an ecological "silent third partner" mutualistic role for seed dispersers [**20**] similar to an increasingly recognized interaction between microbes and pollinators [**21**].

Here, we characterize the availability of ethanol in ripe fruits consumed by chimpanzees at two field sites across the species' geographical range – at an East African locale inhabited by Eastern chimpanzees (*Pan troglodytes schweinfurthii*), and a West African site inhabited by Western chimpanzees (*P.t. verus*). We then estimate daily rates of ethanol ingestion by chimpanzees, and show that these rates are comparable to contemporary drinking patterns in modern humans.

1.2 Materials and Methods

1.2.1 Study sites

In East Africa, fruit-ethanol data were collected at the site of the Ngogo Chimpanzee Project [**22**] (Ngogo; N 0°29'55", E 30°25'30"; altitude ~1400 m) in Kibale National Park, Uganda, over two separate field seasons from October 2017 to June 2018 and August to December 2019. To conduct this work, permits were obtained from the Uganda National Council for Science and Technology (UNCST, permit NS41ES) and the Uganda Wildlife Authority (UWA). In West Africa, fruit-ethanol data were collected at the site of the Taï Chimpanzee Project [**23**] (Taï; N 5°52'4",W 7°20'21"; altitude ~200 m), Taï National Park, Côte d'Ivoire, from June to September of 2021. To conduct this latter work, a permit was obtained from the Office Ivoirien des Parcs et Réserves of Côte d'Ivoire. Additional data pertaining to chimpanzees used in this study were obtained from previously published studies; the researchers had no direct contact with chimpanzees during the course of this work and thus no institutional animal approvals or animal research permits were needed.

Continuous, long-term research on the Ngogo chimpanzees began in 1995, and at the start of this study, the community there was the largest group of chimpanzees ever observed with

over 200 individuals [**22**]. During this study, the community split in January 2018 [**24**]. The Ngogo chimpanzees are ripe-fruit specialists, spending the highest percentage of annual feeding time on *Ficus mucuso* (~18%) [**25**], a relatively large fig obtained primarily in the canopy [**26**]. The daily minimum and maximum temperatures, collected at the site, averaged 17.0 and 25.1°C, respectively, during the first field season, and 16.8 to 23.0°C during the second field season.

Taï is home to four habituated communities of chimpanzees, each typically composed of 20–40 individuals, with the North Group having been studied since 1979 [**27**]. Chimpanzees at Taï are followed and monitored daily by field assistants, volunteers, and researchers and their diet is well-characterized. Taï chimpanzees of the North Group spend the majority of their time (12.5%) feeding on *Parinari excelsa*, and 7.5% of their time on *Sacoglottis gabonensis* [**28**], both of which are consumed almost exclusively on the ground. The daily minimum and maximum temperatures collected at the site during the study period averaged 24.4 and 28.3°C, respectively.

1.2.2 Sample collection

Fruit samples were collected opportunistically from crops observed being consumed by chimpanzees. In the case of *Parinari excelsa*, recent chimpanzee feeding was confirmed for each crop using camera traps (GardePro A3, Hong Kong, China). Substantially damaged or partially eaten fruits were not collected, with the exception of *Musanga cecropioides* at Taï, which could only be collected as freshly broken pieces dropped from the canopy by chimpanzees. For fruit species consumed exclusively in the canopy, samples were only collected if we had witnessed the fruit fall or otherwise inferred that the fruit had recently fallen, e.g., by wet sap on the stem; visibly oxidized and aging fruits or fruits that had been on the ground for an indeterminate period were excluded. Fruits consumed by chimpanzees exclusively on the ground were likewise collected only if they appeared recently fallen, via examination of the portion of the fruit touching the ground; fruits that were moldy, saturated with mud, or embedded in the ground were avoided. Samples were double-bagged, transported to the field lab, and then frozen in a -5 °C freezer to arrest fermentation until subsequent analysis. Fruit transit times from fruit crop to field lab were minimized and varied depending on distance to camp, averaging ~two hours.

Estimates of fruit ripeness were determined in the field at both Ngogo and Taï, by researchers and field assistants with extensive experience identifying and classifying ripe fruit ingested by chimpanzees. Typically, the individual fruits of the fruit crops we studied developed and ripened more or less synchronously, and the transition in ripeness status of most species was clearly identifiable, signaled by species-characteristic changes sensible to humans, e.g., changes in color, an increase in mass, and becoming softer when palpated. Some fruits among otherwise ripe fruit crops nonetheless occasionally stood out as unripe or underdeveloped and was labeled as such. And at Ngogo, we repeatedly observed chimpanzees briefly eating small crops of crunchy deep-green obviously unripe figs, presumably as a fallback dietary strategy in the absence of higher quality food. All such fruits, classified as unripe, were excluded from this study.

During the first field season at Ngogo, we collected several fruits ingested by chimpanzees, resulting in relatively small samples of each species. During the subsequent field seasons, we prioritized collection of the aforementioned top fruit species consumed by chimpanzees at their respective sites, namely *F. musuco* at Ngogo, and *P. excelsa* and *S.*

gabonensis at Taï, with any other available species sampled haphazardly in smaller quantities. Only species with at least three ripe fruit samples were included in the analysis.

1.2.3 Fruit ethanol assays

We used three methods to assay fruit ethanol concentrations, developed sequentially during three field seasons (described individually below). In 2017 – 2018 at Ngogo, we used only a metal oxide semiconductor (MOS) sensor to estimate gaseous ethanol within fruit slurry headspace. This method and the overall experimental approach were based loosely on [**29**]. In anticipation of returning to Ngogo in 2019, we successfully piloted another very similar method, but instead using a field-portable gas chromatograph (GC) that utilizes a micro-electro-mechanical system sensor to estimate concentrations of gaseous ethanol within fruit slurry headspace. Both the MOS and the GC methods require careful time-consuming lab work, and to streamline logistical constraints in the field the MOS sensor was not used in 2019. However, the GC instrument proved very sensitive to the tropical humid air (discussed below). Thus, in anticipation of a third field season at Taï, we successfully piloted a different non-headspace assay method, this time using a chemical assay kit based on dichromate (Cr_2) as the primary reagent, which changes color in proportion to its reaction to ethanol. To validate these methods, a subset of fruits at Taï were also analyzed using the MOS method. Despite attempts to similarly validate the GC method, this instrument entirely failed to operate at Taï due to the persistently high levels of humidity (>90%), which were significantly less pronounced at Ngogo.

All methods (described in greater detail below) required initial preparation of fruit slurry from defrosted samples. Exocarp and seeds were excluded whenever these were not consumed by chimpanzees. For species of *Ficus*, seeds and exocarp were always included in the assay. Fruits were cut into longitudinal sections, inserted into a 50 ml centrifuge tube and homogenized into a slurry using a battery powered homogenizer (14 mm tip diameter; Tissue-Tearor, Biospec Products, Bartlesville, OK, USA). To convert each method's raw data outputs to ethanol concentrations, we made calibration standards by serially diluting 70% ethanol with purified water. Calibration standard series were then used to create linear regressions, the slope and intercept of which were employed to predict the ethanol concentration of each associated fruit sample. Negative values were rounded up to zero. Ethanol concentrations were always calculated in units of percent mass, namely weight over weight (% w/w). All equipment in contact with fruit slurry was sterilized prior to each use by soaking in 1% bleach solution for 15 minutes.

1.2.4 Statistical analyses

To determine whether parametric or non-parametric statistics should be used to compare groups, we first assessed the normality of each group, or the normality of the residuals of analyses of variance (ANOVA). Normality was checked using the Shapiro-Wilk test of normality ('shapiro.test' function in the 'stats' package) in R (v4.4.2) [**30**], as well as by examining the histogram using the 'hist' function and the QQ-plot using the 'ggqqplot' function. Differences between pairs of groups were then assessed using either a Student's t-test ('t.test' function) or the non-parametric Mann-Whitney U-test ('wilcox.test' function), accordingly; all tests were two-tailed. Differences between multiple groups were assessed using ANOVA ('aov' function), or the non-parametric Kruskal-Wallis test ('kruskal' function in the 'agricolae' package).

Phylogenetic effects on average species ethanol concentrations were examined using a phylogenetic linear mixed model (PLMM) approach, using the 'pglmm' function in the 'phyr' package [**31**]. The species phylogeny was obtained using the 'phylo.maker' function of the 'V.PhyloMaker2' package in R [**32**]. Two models of ethanol concentrations were compared, one a linear mixed model incorporating only species identity as a random effect, and another that added phylogenetic relationships as another random effect. Additional coefficient of determination estimates associated with each model were made using the 'R2' function in the 'rr2' package [**33**], using the R^2 value associated with maximum likelihood (R^2_{lik}) for comparison. This paired model approach was repeated across datasets with combinations of each method and field season separately, as well as pooled.

1.2.5 Metal oxide semiconductor (MOS) sensor method

This method utilized a vapor alcohol sensor (TGS2620, Figaro USA, Inc., Arlington Heights, IL, USA; ETH-BTA, Vernier Software and Technology, Beaverton, OR, USA) connected to a touchscreen interface (LabQuest 2, Vernier Software and Technology) and datalogger software (LoggerPro 3.15, Vernier Software and Technology) to sample alcohol concentrations within the headspace above fruit slurry. Electrical conductivity of the sensing chip increases when alcohol vapors contact the internal metal oxide semiconductor layer. The sensing chip is recommended for "alcohol testers" and "organic vapor detectors/alarms" by the manufacturer (TGS2620, Figaro USA) and therefore although the instrument is "very sensitive to ethanol vapor" it can also respond to other organic volatile compounds (ETH-BTA, Vernier Software and Technology). Fruit samples analyzed using this method were first diluted 1:1 with purified water to facilitate homogenization. Exceptions to this dilution factor included two samples of *Phytolacca dodecandra* and one sample of *Uvariopsis congensis* which were analyzed undiluted during early sampling at Ngogo, and which were sufficiently watery to not require dilution. At Taï, seven samples of *Saccoglottis gabonensis* were diluted three-fold and one sample of *Parinari excelsa* four-fold to match the dilution factor of the accompanying Cr_2 method during cross-validation (see below). Five grams of fruit slurry (or calibration standard) were pipetted into a 50 ml self-standing centrifuge tube, which was then sealed with a non-porous oven bag plastic to allow gas equilibration between sample and headspace. To record output voltage, we made a cut in the plastic and inserted the alcohol sensor 1 cm above the slurry until the voltage remained constant for 20 s.

For fruits assayed using this method at Ngogo in 2018, headspace was equilibrated for 15 minutes prior to sample collection. The instrument was pre-calibrated in the lab prior to arrival at Ngogo by pooling multiple serial dilutions ranging from 1.6% to 0.0125% ethanol, across n=27 standards. Because this method can be sensitive to temperature, we documented the internal temperature of each calibration standard in the lab using a stainless steel temperature probe (Vernier Software and Technology); values averaged 22.9°C (range 22.0–23.8°C). While at Ngogo, we likewise recorded all fruit sample temperatures, cooling the sample tubes in ice water or warming them using body heat as needed, which resulted in an average value of 22.4°C (range 21.3–23.1°C). At Taï in 2021, headspace was equilibrated for up to 8 hours, with calibration standards being analyzed alongside samples and thus eliminating the need to keep track of temperature. Rather, four series of calibration standards were prepared by serial dilution, composed of 0.8, 0.4, 0.2 and 0.1% ethanol, and were assayed alongside four series of fruit samples representing n=71 fruits; another three calibration series associated with a total of 11

fruits did not contain the 0.8% standard. The factory calibration of this instrument defaults to a power regression, and we therefore log-transformed the known ethanol concentrations of our calibration standards to obtain a linear regression for the series. Resulting fruit ethanol values were then exponentially transformed and multiplied by the dilution factor.

To assess the effect of headspace equilibration time with fruit slurry on ethanol concentration measurements using this method, we separately assayed samples from the same pool of fruit slurry over increments of 0.25, 0.5, 1, 2, and 4 hours using three *Sacoglottis gabonensis* fruits at Taï. No significant differences in ethanol concentrations were observed within the headspace of these samples (repeated measures ANOVA, $F_{4,8}$=1.7, p=0.245). Fruit-ethanol concentrations over time were consistent and differed from the mean of each sample on average by 8%. Differences over 4- and 8-hour increments using another three samples from the same species were nearly significant (Student's paired t-test, t_2=3.9, p=0.059), with all three decreasing by an average of 8% of the 4-hour value (**Supplementary figure S1.1**, see **1.5 Appendix**). Using this data we also estimated the mean absolute error (MAE) associated with repeated sampling of the same fruit slurry using this method, which averaged 0.004% (ranging 0.001 to 0.008%) relative to an average ethanol concentration of 0.10% (ranging 0.02–0.18%).

1.2.6 Portable gas chromatograph (GC) method

A portable gas chromatograph (SeaPORT Mini-GC, Seacoast Science, Inc., Carlsbad, CA, USA) was used to measure ethanol concentrations of headspace over fruit slurry at Ngogo in 2019. This device separates constituent gases by molecular weight and exposes them to a micro-electro-mechanical system sensor that produces a corresponding voltage output over time. Thus, in principle other short chain alcohols such as methanol and isopropyl alcohol could inflate an ethanol peak, although these other alcohols are not typically major products of fermentation. The gas chromatograph interfaced with the same software as the aforementioned MOS method (namely, Logger Pro 3.15); ambient air was used as a carrier gas. Samples were prepared by evenly applying 1.5 g of fruit slurry to a 30 mm petri dish, covered with non-porous plastic oven bag material and rubber bands, resulting in 3.5–4 ml of headspace. We then used a gastight syringe (1001 LT SYR with Kel-F hub point style 2 needle, Hamilton Company, Reno, NV, USA) to pierce the plastic covering of the petri dish to extract 1 ml of headspace for injection into the gas chromatograph. All headspace measurements were collected at a continuous column temperature of 40°C and pressure of 7 kPa. Samples were not diluted during this field season, as all fruit species chosen for analysis contained sufficient water content to not require it.

To interpret the ensuing GC signal, we used the 'Integral' tool in Logger Pro to estimate the area under each peak (in units of mV*min). Based on observations of ethanol calibration standards obtained from the instrument in the field, initiation of a fruit sample ethanol peak was expected to take place 50–54 seconds after sample injection. To reduce error due to a delayed return to baseline after a signal peak, we estimated the time of end of each peak as when the signal decreased to 1.2 mV above baseline.

Two series of calibration standards, composed of 2.4, 1.2, 0.6, 0.3, and 0.15% ethanol, were analyzed, one immediately before and one after each series of fruit samples, using separately prepared standards. Due to this instrument's sensitivity to water and its use of ambient air as a carrier gas, samples could only be analyzed on the GC during times when the ambient relative humidity at Ngogo fell below ~60% during mid-day. Because of these logistical and

timing constraints, samples were prepared the night before and stored in a cool insulated place, with resulting equilibration times ranging from 17–24 hours (20-hour average). Samples that could not be analyzed within a day were discarded. To prevent fermentation during the equilibration process, all fruits were frozen in a -5°C freezer prior to sample preparation, and all equipment was sterilized using 1% bleach solution.

1.2.7 Dichromate (Cr_2) reduction method

The dichromate method utilized two reagent kits, one for precipitating saccharides in a liquid sample (DRSK-500, BioAssay Systems, Hayward, CA, USA), and the other for colorimetric estimation of ethanol based on a reduction reaction with dichromate, whose formula is optimized to reduce "interference by [non-ethanol] substances in the raw samples" (DIET-500, BioAssay Systems). While the kits were formulated for samples such as "wine, beer, culture media… etc." they have not been validated for assaying fruit ethanol. The kit recommends deproteinating protein-rich samples, which we did not do because fruits are not typically considered to be protein-rich, samples were diluted at least two-fold prior to assay, and the solid mass of the fruit was centrifuged out prior to assay, which would naturally filter out most proteins from the assay supernatant.

Each fruit sample was homogenized after dilution with 1:1 purified water, weighed to 1.5 g within a 1.5 ml centrifuge tube, and centrifuged for 15 minutes (10K Mini Centrifuge, Qor Labs) to obtain 300 µl of supernatant. If under 300 µl was produced, slurries were re-diluted as needed, up to a ratio of 1:4. We then added saccharide-precipitating reagents to both the fruit samples and the calibration standards, waited 15 minutes for incubation, centrifuged for 5 minutes, and extracted 400 µl of the resulting supernatant. To this supernatant, we added the assay start reagents sequentially, incubated for 30 minutes, and then added the stop reagent. We then used a field portable cuvette reader (GDX-SVISPL, Vernier Software and Technology) and associated software (Spectral Analysis, version 4.8.4-1356, Vernier Software and Technology) to measure color absorbance at 580 nm. To make the resulting values comparable to the previously described headspace methods, which estimate the ethanol concentration of the whole fruit rather than just of the supernatant, we also determined the dry mass of the remaining centrifuged fruit pellet. Pellets were put in weigh boats, air-dried in the sun, and placed in plastic bags with large volumes of silica gel. Desiccation time in silica gel bags was determined by repeated weighing of the pulp until it no longer decreased in mass (~72 hours). Dry mass was divided by total centrifuge mass and multiplied by the dilution factor to obtain the dry mass percent of the fruit.

Calibration standard cuvettes were read before and after each series of fruit samples, with each series of standards composed of a serial dilution of 0.8, 0.4, 0.2, and 0.1% ethanol. Bookending the sample assay with calibration standards was necessary, because the cuvette reader contains a lamp that is sensitive to changes in temperature and humidity over time, which at times fluctuated significantly at Taï. Ethanol concentrations of fruit samples were determined by multiplying the absorbance by the slope of the calibration series regression, plus the intercept. These latter values were then multiplied by the dilution factor and by one minus the dry mass.

To estimate the precision of this method, as well as the variation associated with assaying different segments of the same fruit sample, we cut three fruit samples of *Parinari excelsa* into thirds and assayed each segment in triplicate, resulting in nine sets of triplicates. Repeated assays of the nine pools of slurry collected in triplicate resulted in a mean absolute error (MAE) of

0.02% ethanol, relative to an overall mean value of 0.17% (ranging 0.01–0.04% across the nine samples). To separately estimate error associated with different parts of the fruit, three longitudinal segments collected from each of three *P. excelsa* fruits yielded MAE values of 0.01–0.02%, relative to the same overall mean value of 0.17% (**Supplementary figure S1.2**).

1.2.8 Cross-validation of Cr_2 and MOS ethanol assay methods

To evaluate the consistency of ethanol concentrations as determined by the Cr_2 and MOS methods, 51 samples of *S. gabonensis* and 5 samples of *P. excelsa* were assayed simultaneously using both methods at Taï (the GC method failed to operate and was not cross-validated). For *S. gabonensis*, the differences in the average concentrations of the MOS and Cr_2 groups were not significant, averaging 0.27 and 0.30%, respectively (paired Mann-Whitney U-test, V=625, p=0.725), and for *P. excelsa*, these differences were greater than twofold, and nearly statistically significant (averaging 0.19 and 0.46%, respectively; paired Mann-Whitney U-test, V=0, p=0.063; **Supplementary figure S1.3**). A linear model of *S. gabonensis* methods yielded an adjusted R^2 of 0.04 (p=0.18), however, with one extreme outlier among the Cr_2 group removed (Sg31, identified via the interquartile range method), this value increased to 0.22 (p=0.10); a linear model for *P. excelsa* was not significant (**Supplementary figure S1.4**). Thus, despite nearly identical group means the distribution of the data is less well correlated than might be expected (R^2=0.22; **Supplementary figure S1.4**). To determine whether these differences are within the expected maximum error range of the combined methods, we first calculated the maximum variation of the known versus the predicted ethanol concentrations of the associated calibration standards of each method, and added them together, resulting in an average of 0.09% (or 0.07, 0.13, 0.06 and 0.09% corresponding to calibration standards binned into 0.1, 0.2, 0.4 and 0.8%, respectively). To these we added the maximum variation in estimated ethanol concentrations from repeatedly sampling from the same pool of fruit slurry (**Supplementary figure S1.1 and Supplementary figure S1.2**), which contributed another 0.05% of error, totaling 0.14% maximum error. We then averaged the estimated fruit ethanol values from both methods, assuming them both to be true, and calculated the absolute difference from this average for each value. Fortynine out of fiftyone *S. gabonensis* fruits were under 0.12% error, averaging 0.04%, well under the maximum error range of 0.14%. The two erroneous fruit samples, Sg31 and Sg45, resulted in significantly higher concentrations via the Cr_2 method than the MOS method. However, removal of these outliers has a minimal effect on the overall results and since no such protocol exists for the remaining fruit species, we assumed the Cr_2 method was correct. It is not possible to similarly analyze the maximum error associated with the only *P. excelsa* cross-validation calibration series. However, when compared to the error threshold associated with *S. gabonensis* calibration standards, the error of all the *P. excelsa* samples was within limits. The observed differences among these method results may in part derive from the different physical approaches used to measure ethanol. The Cr_2 method relies on optically sensing changes in color due to a chemical change within a liquid medium on the one hand, and the MOS method detects changes in the electrical conductivity of a semiconductor material in response to contact with ethanol vapors on the other. Each method has several other steps in addition to these, each of which may introduce variability that is unique to the method. Assay estimates may also be influenced by between- and within-species differences in fruit pulp biochemistry and tissue structure, with possibly variable effects on each method. Without further methods validation individual false positive values remain possible, however they are unlikely to

be systematic given that one or more calibration series are associated with samples ranging in value from near-zero to the average ethanol value for each species.

1.2.9 Feeding-time weighted body-mass specific dosage estimates

We obtained weighted averages of ethanol concentrations of fruit species at each field site by scaling their concentration to the reported annual percentage feeding time of that species for Ngogo [**25**] and [**28**] for Taï, based on averages from long-term data collected over a 15- and a 25-year period at each site, respectively. Such annualized fruit feeding times may not necessarily be proportional to the annual mass of consumed fruit mass, as feeding rates associated with individual fruit species can be highly variable. Nonetheless, at Ngogo we collected data for thirteen species, which collectively represent ~46% of annual feeding time spent by chimpanzees across all dietary categories. We divided the percentage of feeding time associated with each species by the total percent, and then multiplied this value by the average ethanol concentration of that species to determine its contribution to the total estimate. These values were then summed to produce a feeding-percent-weighted average.

To convert the percent ethanol to grams ethanol, an estimate of the total daily food mass consumed by chimpanzees is needed. Comprehensive data on daily food mass ingested by wild chimpanzees are scarce and require year-long focal following of multiple individuals, along with careful observation of feeding rates on individual fruit species. The only such published study was conducted at a field site 10 km from Ngogo (i.e., Kanyawara), where female and male chimpanzees were found to consume 6.0 and 6.3 kg of food (including fruit) each day, respectively [**34**]. Here we apply these data to estimate consumption by chimpanzees at Ngogo and Taï, respectively, and note that such feeding behavior may vary from site to site due to differing rates of caloric expenditure and the overall caloric density of available food. Ngogo and Taï chimpanzees spend comparable amounts of time feeding on fruit (71% vs. 76%) [**25**,**28**], and these proportions were used to estimate total fruit mass consumed daily at the two sites. These values were multiplied by the weighted ethanol percentages to estimate ethanol dosage in grams for males and females across both sites.

To further estimate body-mass specific ethanol dosage between individuals of different body sizes, we divided grams of pure ethanol by the estimated body masses (kg) of wild female and male chimpanzees of the two subspecies in this study, *Pan troglodytes schweinfurthii* and *Pan troglodytes verus*. Body masses were estimated from seven adult females and six adult males of *P. t. schweinfurthii* [**35**] and from one male and three females for *P. t. verus* [**36**]. Resulting dosages, in units of grams ethanol per kilogram body mass per day of feeding (g/kg/day), were then compared to that of a single standard drink consumed by a 70 kg human. The most commonly used value for a standard drink internationally is 10 g of pure ethanol [**37**]. Chimpanzee dosages in grams per kilogram are then divided by the resulting human value of 0.14 g/kg per standard drink, to estimate the daily equivalent of standard drinks consumed by chimpanzees.

1.3 Results

1.3.1 Feeding-time weighted percent ethanol

Ethanol concentrations were estimated for 106 ripe fruit samples belonging to 13 angiosperm species consumed by Eastern chimpanzees at Ngogo in Uganda in 2017 – 2018, using the metal oxide semiconductor (MOS) method (see **1.2 Materials and Methods**). The linear regression derived from calibration of this instrument yielded an adjusted R^2 of 0.94 ($p<0.001$) and a Mean Absolute Error (MAE) of 0.07. The taxa assayed correspond to ~65% of the annual time spent feeding on fruit by chimpanzees at this site [**25**] (**Figure 1.1**). Scaling species-level means for all thirteen species by the corresponding annual fractional feeding time by Ngogo chimpanzees [**25**] resulted in an average ingested ethanol concentration of 0.35% (weight/weight for all ethanol percentages). Nearly 60% of this value is derived from fruits of *Ficus mucuso*, due to its relatively high ethanol concentrations (0.53%, $n=15$) and the high annual fraction of feeding time spent on this species (18%). The remaining fraction of ingested ethanol reflects contributions from the eight next most frequently consumed fruit species in the study sample. The second-most consumed fruit species at Ngogo (*Uvariopsis congensis*) composes 10.0% of feeding time, and with an average ethanol concentration of 0.13% ($n=8$).

Ethanol concentrations were separately estimated for 148 ripe fruit samples belonging to 6 angiosperm species the following year at Ngogo, corresponding to ~41% of the annual feeding time, and including four species sampled the previous year (**Figure 1.1**). These fruits were assayed using the gas chromatograph (GC) method; eleven separate calibration series were used to estimate the ethanol concentrations within eleven corresponding batches of fruit samples, resulting in linear models with adjusted R^2 ranging between 0.65–0.92 (0.77 average; all $p<0.003$) and MAE ranging 0.19–0.34 (average 0.29). These species resulted in a weighted average of 0.36%, with *F. mucuso* representing 67% of the value.

Pooling sample data across assay methods and field seasons at Ngogo, with 254 fruits across 15 species corresponding to ~79% of annual feeding time, resulted in a weighted ingested ethanol concentration of 0.32%. *Ficus mucuso*, which averaged a pooled concentration of 0.41%, contributed 44% of the weighted value, with the following nine species composing the rest.

Among Western chimpanzees at Taï in Côte d'Ivoire, ethanol concentrations were estimated for ripe fruit samples ($n=245$) from six angiosperm species, which correspond to 32% of the annual time that Taï chimpanzees spend eating fruit [**28**] (**Figure 1.1**). Of these, 225 samples from 5 species were analyzed using the dichromate (Cr_2) method; seventeen calibration series were paired with their respective samples, resulting in linear regressions with adjusted R^2 values ranging between 0.95–1.00 (all $p<0.001$) and MAE values ranging over 0.00–0.05. Another 17 samples from two species of fig were assayed using the MOS method (11 samples from *F. recurvata* and 6 from *F. saussureana*), alongside an additional 56 samples of two species assayed using both the Cr_2 and the MOS methods for cross-validation (51 samples from *Sacoglottis gabonensis* and 5 from *Parinari excelsa*). In all, a series of seven MOS calibrations resulted in linear regressions with adjusted R^2 values ranging 0.98-1.00 (all $p<0.045$) and MAE ranging 0.01–0.03. Taï chimpanzees spend more time consuming *P. excelsa* than any other food item, which composes 12.5% of total annual feeding time; ethanol concentrations for fruits of this species averaged 0.40% ($n=149$). The second-most consumed fruit species, *S. gabonensis*, averaged 0.30% ethanol ($n=51$). The weighted average ethanol concentration among all six species (with two species of *Ficus* pooled as *Ficus* spp.) was 0.31%; 97% of this weighted average is represented solely by *P. excelsa* and *S. gabonensis*.

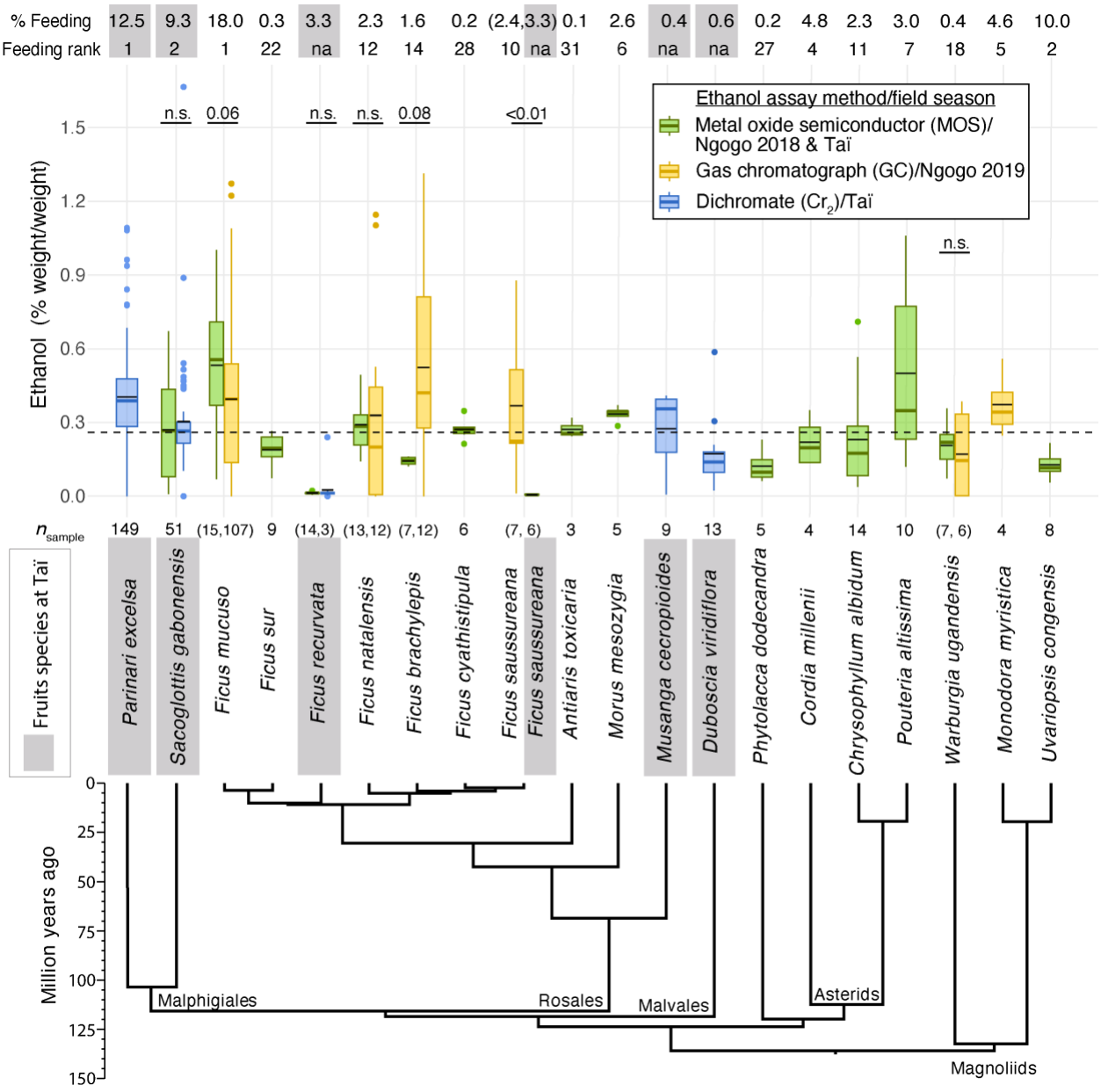


Figure 1.1: Average ethanol concentrations of fruits at Ngogo and Taï. Taxonomic names of fruit species are arranged according to their evolutionary relationships [**32**], with fruits from Taï highlighted in grey. Boxplots follow standard interquartile range notation and are color-coded by ethanol assay method, as associated with the field seasons specified in the embedded legend. The thicker color-coded line within each box represents the median, and the thin black line represents the mean. Statistical notation above species assayed using two methods indicates the results of a Mann-Whitney U-test (unpaired for all species except *Sacoglottis gabonensis*; V=625 and W=1047, 56, 93, 21, 0, and 23, respectively), and are labeled either as n.s. (not significant), or with a p-value for species at or near the fiducial threshold of 0.05. A dashed horizontal line across the plot indicates the overall pooled species average of ethanol concentrations (0.26%). The top two rows of numbers indicate the annual percentage of time spent feeding on each species by chimpanzees, along with their feeding ranks among all fruits (including figs) at either Ngogo [**25**] or Taï [**28**]. Annual feeding percentages for the two *Ficus* species at Taï (representing 3.3% of annual feeding time) were pooled. Sample size (n) for each cluster of fruit is indicated above the name of each species.

Phylogenetic relationships did not play a role in explaining ethanol concentrations for any combination of field seasons/methods. Species identity on its own explained a modest amount of variation in ethanol concentrations for six species collected using Cr_2 and MOS methods at Taï (R^2_{lik}=0.26), and thirteen species collected using the MOS method at Ngogo in 2018 (R^2_{lik}=0.24). Species identity did not explain any variation in ethanol values for six species collected using the GC method at Ngogo in 2019 (R^2_{lik}=0.03).

1.3.2 Body-mass specific dosage estimates

We estimated fruit ingestion rates across sexes and field sites using values from published literature [**34**] (see Methods; Ngogo females: 4.3 kg/day; Ngogo males: 4.5 kg/day: Taï females: 4.6 kg/day; Taï males: 4.8 kg/day). Using the weighted ingested ethanol concentration of 0.32% for pooled data at Ngogo, and 0.31% for Taï, the corresponding rates of ethanol range from 13.6–15.0 g/day for male and female chimpanzees. Body-mass specific estimates of ethanol ingestion are much higher, however, because chimpanzees are smaller than humans. The mass-specific dosage for a 35.9 kg female of the Eastern chimpanzee subspecies corresponds to the equivalent of 0.38 g/kg/day dosage (±0.24 standard deviation), which is the mass-equivalent of 2.6 standard drinks (±1.7) daily for a 70 kg human (**Table 1.1**).

1.4 Discussion

Average ethanol concentrations of chimpanzee-consumed fruit, as weighted by the percentage of feeding time that each species is consumed in nature, were consistent across sites (i.e., 0.32% when pooled across two field seasons at Ngogo, and 0.31% at Taï). These values are somewhat higher than the mean interspecific concentrations at each site, namely 0.29% (n=15) and 0.18% (n=6), respectively. Moreover, the most frequently consumed fruit species (on an annual basis) at each site contained some of the highest overall concentrations across the dataset (i.e., 0.41% ±0.31 for *Ficus musuco* as pooled from two field seasons at Ngogo, and 0.40% ±0.19 for *Parinari excelsa* at Taï; **Figure 1.1**). Given the large variation in ethanol concentrations associated with these fruit species, including some samples with zero or near-zero ethanol content, chimpanzees could potentially be behaviorally avoiding consumption of fruits with higher concentrations. Conversely, if chimpanzees are attracted to ethanol as we had originally hypothesized, then they could potentially consume fruits with higher concentrations and thereby increase their daily ethanol dosage. All ethanol concentrations are reported here in units of alcohol by mass, but due the low density of ethanol, conversion to the more familiar units of alcohol by volume (ABV) would result in ~30% higher concentrations. These overall levels of ethanol are consistent with data from multiple previous studies which suggest that fruit ethanol content is fairly low and typically < 1% [**18**,**38**]. In Panama, average ethanol concentrations of ripe pulp averaged 0.5% in the palm *Astrocaryum standleyanum* [**29**,**39**], and averaged 1–2% for ripe hogplums (*Spondias mombin*) which were partially consumed and dropped by spider monkeys [**40**]. By contrast, ethanol in floral nectar of the bertam palm (*Eugeissona tristis*) consumed by pen-tailed tree shrews (*Ptilocercus lowii*) and slow lorises (*Nycticebus coucang*) in Malaysia averaged 0.6%, but ranged as high as 3.8% [**41**]. The relevance of all such fruit-ethanol concentrations depends, in part, on the rate and extent of consumption by the frugivore. Given

the substantial daily fruit masses consumed by chimpanzees in the literature, the concentrations we report correspond to substantial chronic levels of ethanol intake (i.e., 14–15 g/day).

Table 1.1: Estimated daily body-mass specific ethanol dosage. Fruits compose 71% of the diet of chimpanzees at Ngogo [**25**] and 76% at Taï [**28**]; these values were multiplied by the bulk food estimates to calculate the daily mass of fruit consumed. Ethanol concentrations were weighted by estimated percent annual time chimpanzees spend consuming each fruit species [**25**,**28**] (see **1.2 Materials and Methods**), and grams of pure ethanol were derived by multiplying the concentration by the estimated daily mass of fruit consumed. Dosage corresponds to grams of pure ethanol divided by estimated chimpanzee body mass. The equivalent number of standard drinks were calculated by dividing chimpanzee ethanol dosage by 0.14, i.e., the ratio of 10 g ethanol contained in one standard drink [**37**] over 70 kg human body mass.

Site	Sex	Body mass (kg)*	Bulk food (g) †	Fruit consumed (g)	Ethanol % w/w (± SD)	Ethanol (g) (± SD)	Dosage (g/kg/day) (± SD)	Standard drinks (± SD)
Ngogo	F	35.9	6020	4274	0.32 (0.20)	13.6 (8.7)	0.38 (0.24)	2.6 (1.7)
Ngogo	M	42.0	6333	4496	0.32 (0.20)	14.3 (9.2)	0.34 (0.22)	2.4 (1.5)
Taï	F	41.6	6020	4575	0.31 (0.21)	14.0 (9.6)	0.34 (0.23)	2.4 (1.6)
Taï	M	46.3	6333	4813	0.31 (0.21)	15.0 (10.1)	0.32 (0.22)	2.2 (1.5)
Human reference ‡		70.0				10.0	0.14	1.0

*Body masses of chimpanzees based on reference [**35**] for Ngogo and [**36**] for Taï.

†Bulk food estimates are calculated from reference [**34**].

‡A human reference consuming one standard drink [**37**].

Importantly, estimated body mass-specific dosages for both female and male chimpanzees are high compared to those typically experienced by modern humans (**Table 1.1**).

These results provide the first estimates of daily ethanol intake via frugivory by wild chimpanzees, but we note some important caveats. First, we used a different ethanol assay method during each field season. While this helped ensure a broad assessment of ethanol, it limited comparability of data across field seasons. Future studies would best utilize at least two methods to assay large samples of each species, as was done here with *S. gabonensis* using a headspace and a reagent-based method. The fuel cell breathalyzer headspace assay method, used originally in [**29**] and more recently in [**42**], may serve as an additional source of validation. The field-portable methods used here can also be compared to lab-based approaches such as enzymatic assays and gas chromatography-flame ionization detection (GC-FID), with the goal of better determining pulp-ethanol concentrations in the field. Second, estimates of daily ethanol dosage for chimpanzees (**Table 1.1**) were based on body mass and dietary data obtained from prior research. Future comprehensive studies of dietary ethanol ingestion could incorporate year-long observations of individual chimpanzees with information regarding the specific food items these individuals consume. Third, since animals are expected to first consume the most highly desirable fruits, we likely disproportionately sampled the less desirable fruit specimens. If chimpanzees and other animals are attracted to ethanol as originally hypothesized, this effect would result in conservative estimates for daily ingestion rates. Future studies may artificially exclude frugivores from some crops to evaluate fruit-ethanol values in the absence of animal foragers.

Accurately assaying fruit-ethanol concentrations to test ecological hypotheses is a non-trivial task. Ethanol concentrations were recently estimated for a wide variety of fruit species in Costa Rica, with the conclusion that fruits with mammal-dispersed seeds (as defined by larger fruit size) have higher ethanol concentrations than those that are bird-dispersed [**42**]. This study used vapor sampling via a breathalyzer from fruits placed within a plastic bag (for one hour), in a method similar to that in reference [**29**], and to the headspace methods used here (MOS and GC). Reference standards of 5 ml water/ethanol mixtures were used for calibration. However, because fruits ranged from 1.2 – 149 grams and were studied whole without extraction of the pulp or removal of the rind, the surface area for ethanol diffusion into the airspace may have varied considerably relative to the reference standards. The surface area and rind thickness of fruit samples in [**42**] were not reported, and resulting ethanol vapor measurements from surrounding headspace may underrepresent internal concentrations of ethanol. Despite this, the overall range of ethanol concentrations reported for wild fruits are broadly consistent with our results, providing further support for ethanol being commonplace in tropical fruits albeit at relatively low values.

Mammalian behavioral responses to ethanol exposure are not well characterized. In some cases, ethanol levels above one percent may deter feeding, as in Egyptian fruit bats [**38**,**43**]. Other mammals may, however, be attracted to higher levels. Captive elephants in South Africa preferentially select marula fruits (*Sclerocarya birrea*) with higher sugar concentrations, based on the olfactory sensing of associated ethanol (along with ethyl acetate, an ester of acetic acid) within the fruit headspace [**44**]. The nectarivorous aye-aye (*Daubentonia madagascariensis*) and slow loris (*N. coucang*) both prefer progressively increasing ethanol concentrations (up to 4–5%) in artificial nectar [**45**]. Among the great apes, captive chimpanzees (*Pan troglodytes*) and

orangutans (*Pongo* sp.) voluntarily consume fruit juice containing 10% ethanol [**46**], and wild chimpanzees habitually pilfer human-collected fermented palm sap [**47**].

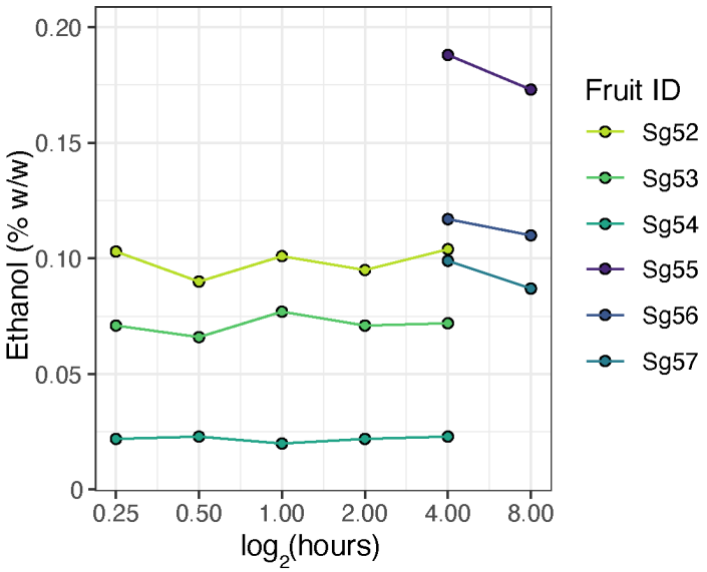
Chimpanzee foraging and social behavior can be influenced by the presence of ripe fruit crops, and by implication of ethanol. Crops of the fig *Ficus mucuso* at Ngogo attract large groups of chimpanzees [**26**], which in turn results in increased social interactions for both sexes, and in social activities such as territorial boundary patrols and hunts [**48**,**49**]. Moreover, initiation of territorial patrols at Ngogo is positively correlated with male party size [**50**], and occurs during periods of high fruit availability and consumption [**51**]. Chimpanzee consumption rates of fruit can be variable through the day, and may include binge feeding. Ethanol exposure can therefore be correspondingly high – ingestion of ~75 ripe figs in a single feeding bout would yield 10 grams of ethanol, although the behavioral consequences of such dosage are unknown.

Similarly, our estimates for body mass-specific ethanol dosage via frugivory are high (**Table 1.1**), but the efficiencies of metabolic clearance pathways in chimpanzees are unknown. Haplorrhine primates (including the great apes) possess at least three copies of *ADH1* that encode the enzyme primarily responsible for ethanol catabolism in the liver, consistent with neofunctionalization [**52**]. Also, an *ADH4* mutation shared by humans, chimpanzees, and gorillas encodes an enzyme responsible for first-pass metabolism of ethanol in the esophagus and stomach, and resulted in a 40-fold increase in catalytic efficiency of this enzyme [**53**]. Catalytic efficiencies for other ADH isozymes involved in ethanol metabolism (or for aldehyde dehydrogenases) have not been characterized for chimpanzees, but may have similarly been targets of positive selection given sustained dietary exposure.

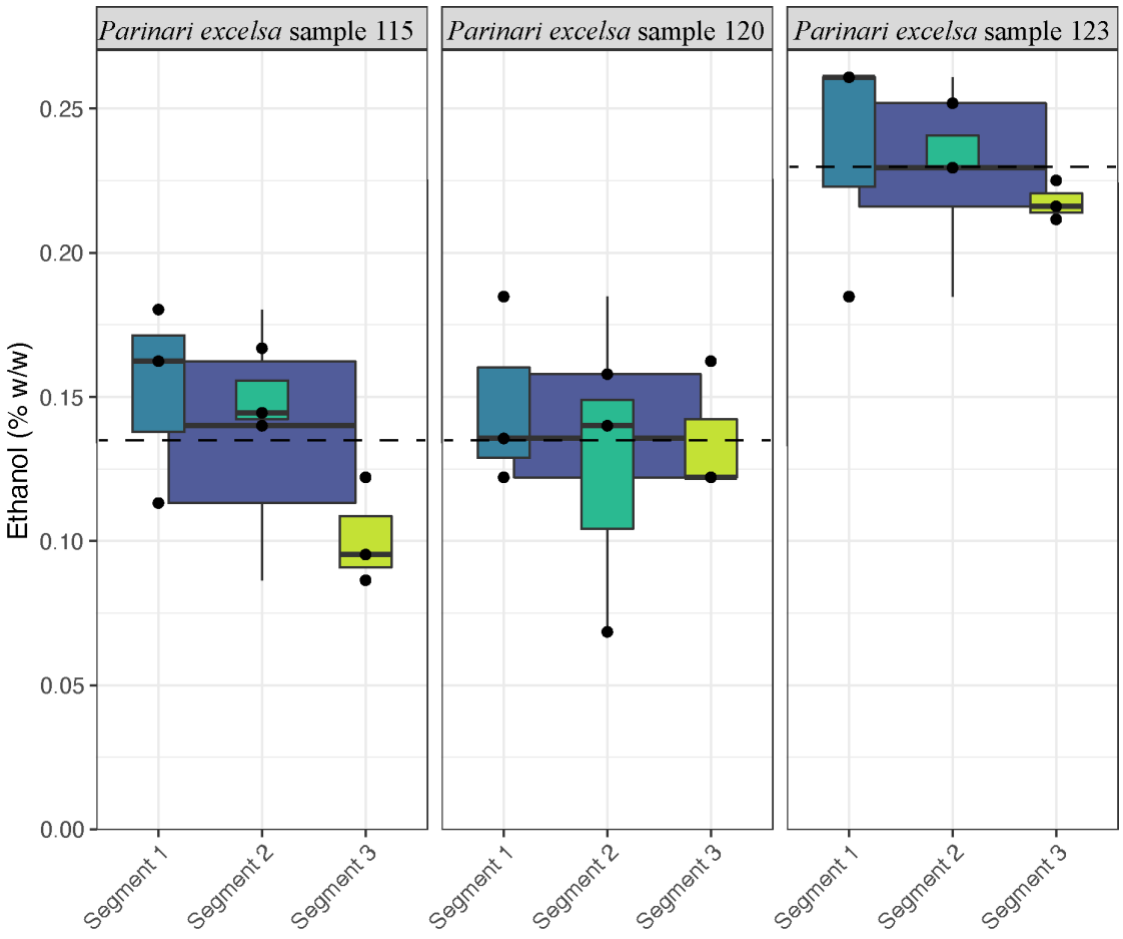
These findings also suggest that the occurrence of non-trivial amounts of ethanol within tropical fruits is widespread, and the diversity of methods and timing by which fermentative microbes disperse onto and into fruits are worth considering. Entry into fruit pulp can occur via multiple modes, including vectoring by insects into fissures in the exocarp. Microbes may also be introduced into the pulp by burrowing insect larvae, and within nectaries of female flowers they may become encapsulated by the developing fruit tissue. Microbes may also enter fallen fruits through microfractures resulting from ground impact, although we found no difference in ethanol concentration between species of fruits typically consumed on the ground (0.27%, n=6 species) versus those consumed in the canopy (0.26%, n=14; Student's t-test, t_{12}=-0.27, p=0.791). For figs, visitation by fig wasps (Agaonidae) provides a means by which yeasts can enter fruits and proliferate at an early stage of development. Fig wasps enter the infructescence (i.e., the syconium), lay eggs in gall flowers, and pollinate the reproductive flowers while simultaneously inoculating the interior with diverse microbes [**54**]. Following eclosion, the next generation of fig wasps (and associated microbiome) then exits the fruit. At Taï, neither fig species contained much ethanol. Twenty fruits of *F. recurvata*, collected the same day and at the same tree, were assayed separately using the Cr_2 method for 6 samples (resulting in 0.05% ethanol) and the MOS method for 14 samples (0.01%). The other fig, *F. saussureana*, contained 0.01% ethanol (MOS method, n=6). In contrast, six species of fig at Ngogo averaged 0.32% ethanol as pooled across methods, including 0.37% ethanol for seven samples of *F. saussureana* (i.e., the same species at Taï). These observations may reflect a starkly contrasting microbial ecology of fig wasps across these two sites (**Figure 1.1**). Regardless of source, proliferating yeasts are commonplace within fruit pulp [**55**–**57**], but the consequences of microbiome composition and fermentation for the evolution of frugivore-angiosperm mutualisms more generally are not well characterized.

Empirical results presented here suggest that chimpanzee dietary exposure to ethanol can be both chronic and considerable. Moreover, these findings are generalizable to other frugivorous primates, and indeed to all tropical vertebrates with a fruit-based diet. Potential roles of ethanol in primate feeding ecology include use of ethanol as a proximate olfactory cue to evaluate fruits suitable for consumption, long-distance sensing of ethanol plumes from fruit for localization, and appetite stimulation increasing rates of fruit ingestion. Ethanol is widespread within fruits of tropical angiosperm species, and the data presented here suggest that its consumption via frugivory is a natural and commonplace phenomenon for frugivores, including human ancestors within Afrotropical forests.

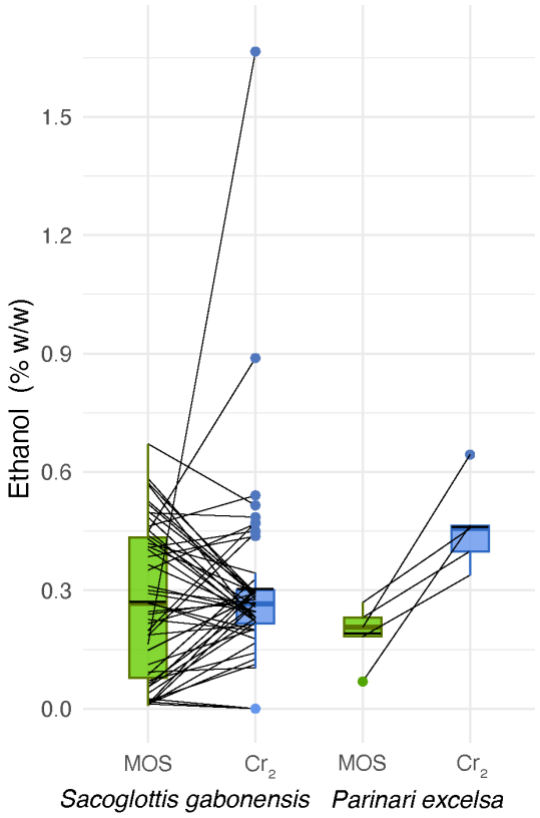
1.5 Appendix



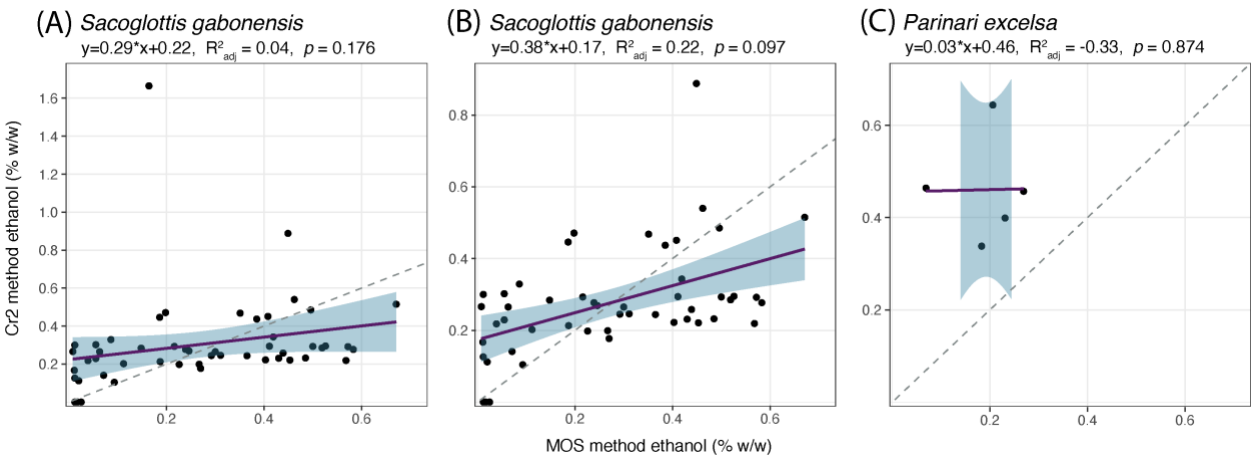
Supplementary figure S1.1: Headspace equilibration timeplot for the MOS assay method. Ethanol concentrations for fruit slurry samples of *Sacoglottis gabonensis* at Taï, collected at incremental headspace equilibration time intervals (time along the *x*-axis is log base$_2$-transformed) using the MOS assay method. Samples each weighed 5 g and were spread out within a surface area of ~28 mm^2 inside a 50 mL self-standing centrifuge tube. Each time point for each individual fruit was prepared separately from the same pool of slurry. No significant statistical differences were observed in ethanol concentrations (see **1.2 Materials and Methods**). These ethanol concentrations were not corrected for the dilution factor implemented when assaying the fruit and are thus not homologous with the other data sets reported, but statistical results are independent of this linear correction. The mean absolute error (MAE) associated with repeatedly sampling of the same fruit slurry averaged 0.004% (ranging 0.001 to 0.008%) relative to an average ethanol concentration of 0.10% (ranging 0.02–0.18%).



Supplementary figure S1.2: Error plot for the dichromate (Cr_2) assay method. Error plot of ethanol concentrations of three *Parinari excelsa* fruit samples from Taï (2021), collected using the dichromate (Cr_2) method. Smaller boxes represent ethanol concentration measurements from three distinct but equal-sized longitudinal segments from each fruit. Larger dark blue boxes in the background represent overall error for a total of nine assays conducted on each fruit (i.e., three assays per segment). Solid lines within each box represent the median and the dashed lines represent the average across all nine samples. The mean absolute error (MAE) from assaying different segments belonging to the same fruit corresponded to 0.01% ethanol, relative to an overall mean value of 0.17%. Segments were not significantly different in ethanol concentration within any of the three samples (One-way ANOVA, $F_{2,6}$=0.4–4.3, p=0.070, 0.699, 0.629, respectively). The MAE associated with each of the nine resulting pools of fruit slurry analyzed separately (i.e., the smaller boxes in front of the dark blue larger boxes above, each assayed in triplicate) was 0.02%, relative to a mean value of 0.17%. Thus the overall MAE for each sample, representing the combined error associated with choosing a random segment and replicates for each fruit sample (corresponding to the larger dark blue boxes), was 0.03%. Ethanol concentrations in this figure were not corrected for either the fruit's dry mass percentage or the dilution factor, but such corrections do not affect the error percentages presented here.



Supplementary figure S1.3: Cross-validation boxplots for the Cr_2 and MOS assay methods. Ethanol concentrations collected using the MOS and the Cr_2 methods for 51 *Sacoglottis gabonensis* and 5 *Parinari excelsa* fruit samples. Thin lines connect values associated with individual samples across boxplots. A thicker green or blue line within each box represents the median value, and a thin black line represents the mean.



Supplementary figure S1.4: Cross-validation regressions for the Cr_2 and MOS assay methods. Cross-validation plots for two methods of ethanol assay (the metal oxide semiconductor (MOS) method and the dichromate (Cr_2) method), using two species of fruit at Taï. Each plot contains a linear regression with 95% confidence intervals, and the slope, adjusted R^2, and *p*-values indicated above each chart. A perfect correlation (i.e., slope of 1) is indicated by a gray dashed line. **(A)** A plot for 51 samples of *Sacoglottis gabonensis*. **(B)** As in **(A)** but with the left uppermost outlier removed. **(C)** A plot for 5 samples of *Parinari excelsa* fruit.

Chapter 2

Urinary concentrations of a direct ethanol metabolite indicate substantial ingestion of fermenting fruit by chimpanzees

2.0 Abstract

Frugivorous animals routinely ingest fruit sugars and the associated products of microbial fermentation. Yeast-derived ethanol within fruit is a recently described chronic feature (~14 g/day) of the chimpanzee diet, but physiological evidence of exposure has not yet been demonstrated. We assayed urine collected from 19 wild chimpanzees for the presence of ethyl glucuronide (EtG), a direct metabolite of ethanol. Urine samples were obtained from study individuals feeding almost exclusively in the canopy of mast-fruiting *Gambeya albida* (=*Chrysophyllum albidum*), freshly fallen fruits of which averaged a pulp-ethanol content of 0.09% (±0.01 SE; range 0.01–0.40%) across multiple ripe crops. Of twenty individual urine samples (nine from females and eleven from males, aged 10–46 years), seventeen tested positive for EtG at an analytical threshold of 300 ng/mL; out of a subset of eleven of these positive samples, ten samples further tested positive at a threshold of 500 ng/mL. These values are high relative to calibrations of the test method for modern humans, and are consistent with substantial rates of ethanol consumption via frugivory in the wild.

2.1 Introduction

Ethanol associated with the fermentation of fruit sugars by endogenous yeasts may be important for the foraging ecology of fruit consumers. As ethanol accumulates within fruit tissues, it can facilitate the localization of ripe fruit crops via long-distance olfaction, help frugivores to assess individual fruit ripeness via sniffing at short distances, and elicit appetitive stimulation as compared to non-fermented fruits [**7**,**18**,**29**,**58**]. For primates, such associations of ethanol with frugivory have been suggested to be the origin of modern-day human attraction to alcohol, with excessive consumption deriving from an evolutionary mismatch between those levels of ethanol available within a primarily frugivorous ancestral diet relative to those available today (i.e., the "drunken monkey" hypothesis) [**6**]. Eastern and western chimpanzees (*Pan troglodytes schweinfurthii* and *P. t. verus*, respectively) have been recently estimated to consume an average daily ethanol dose of 14 g, based on concentrations measured within fruit for major species in their diet along with annualized consumption rates [**59**]. Such a dosage corresponds to ~1.4 standard drinks per day in human terms [**37**]. However, such estimates only provide indirect evidence for substantial ethanol consumption via frugivory.

Multiple methods have been used to more directly assay natural exposure of animals to alcohol. For example, opportunistic postmortem toxicological studies of the liver were used to show that ethanol was the primary cause of mortality in multiple groups of cedar waxwings (*Bombycilla cedrorum*) [**60**,**61**]. Another method involves quantification of ethanol metabolites produced in the liver, especially ethyl glucuronide (EtG), which can be retained in human urine for two to three days [**62**,**63**], and indefinitely within human hair [**64**] and nails [**65**]. Prior work

using mammalian hair [**41**] and bird feathers [**66**] has demonstrated substantial natural ingestion of ethanol among a number of vertebrate species. A study of EtG levels within urine of wild spider monkeys (*Ateles geoffroyi*) in Panama [**40**] found that five out of six monkeys exceeded a clinically meaningful cutoff of 100–200 ng/mL, which for humans is commonly used to monitor abstinence in recovery programs [**63**,**67**–**69**]. Three of these six also exceeded a higher cutoff of 500 ng/mL which is used to rule out the possibility of a false positive due to unintentional alcohol consumption [**63**,**67**–**69**]. Exposure of frugivores and nectarivores to dietary ethanol may thus be widespread, but further characterization is required to understand its effects, if any, on the physiology and behavior of foraging primates.

Assaying EtG within primate urine is thus a feasible method to demonstrate dietary ethanol exposure. Advances in flow immunoassay methods have resulted in a portable dipstick assay for urine EtG, molecules of which compete with enzyme-bound EtG for monoclonal antibody binding sites with concomitant changes in the color of a test strip at a pre-determined threshold [**63**,**70**,**71**]. We assayed chimpanzee urine using such immunoassays in the field, and also determined ethanol content for fruits of a canopy tree species being predominantly consumed by chimpanzee during periods of urine collection. We hypothesized that most if not all chimpanzees feeding on these fruits would pass an EtG assay at a clinically relevant threshold of 500 ng/mL.

2.2 Materials and Methods

Fruit-ethanol content and EtG data were collected at Ngogo, Kibale National Park, Uganda (0°29′55″N, 30°25′30″E) in August 2025. At Ngogo, two large wild communities of eastern chimpanzees (*P. t. schweinfurthii*), which were formerly a single community, have been observed continuously for over 30 years, are well habituated to human presence, and are thus easily monitored (see [**24**]). Each member of the Central and Western communities can be visually identified by long-term researchers and field assistants at the site. At the time of this study these two communities of roughly equal size totaled to ~170 individuals, including 45 adults each as defined by a cutoff of 14 years of age [**72**]. Members of both communities were observed to spend the vast majority of their feeding time consuming the fruit of one species, the African star apple *Gambeya albida* (=*Chrysophyllum albidum*), trunks of which have the highest total basal area (as measured by diameter at breast height) among the top 'high fruit abundance' species consumed by chimpanzees at Ngogo [**73**]. This tree species mast fruits unpredictably every 2–3 years, providing a major spike in caloric gains for Ngogo chimpanzees [**74**,**75**]. Such periods of high food abundance have been previously linked to female reproductive status, which likely explains our observations of a large number of females concurrently in estrus (as indicated by visible anogenital swelling) [**76,77**]. All urine samples were collected and assayed within the same week between August 15–21, 2025. Fruit samples from *G. albida* were collected and assayed for ethanol content during the largely overlapping period of August 9–22, 2025.

Urine from individually identified chimpanzees was collected opportunistically and non-invasively using a pipette from fresh and dry leaf litter. Chimpanzees predictably urinate just prior to leaving a fruit crop and can often be seen preparing to do so by climbing to a lower branch of a tree. Urination on the ground is often accompanied by defecation, and in these cases urine was pipetted from pools in the leaf litter opposite the feces. Samples were only collected on dry days, since rainwater on understory leaves cannot be visually differentiated from urine.

Urine may also be collected directly from underneath urinating chimpanzees by holding out a clean disposable plastic bag placed over the end of a forked branch; urine samples collected from leaf litter were previously shown to not significantly affect measured hormone levels relative to samples collected using a plastic bag [**78**].

Commercially available (and field-portable) monoclonal antibody-based enzyme immunoassays were used to assay urine samples for the liver ethanol metabolite ethyl glucuronide. Assays with two different concentration cutoffs were used, one at 300 ng/mL (Prime Screen DWETG-300, Wondfo USA Co., Ltd., Willowbrook, IL, USA) and one at 500 ng/mL (FelexOSY ETG Urine Test, Innovation Biotech Co., Ltd., Beijing, China); both sets of test kits contained the same basic instructions (albeit using different text and format). Samples were assayed either immediately in the field or following refrigeration at camp upon arrival; urine EtG has been shown to remain stable at room temperature for at least six days [**67**]. The manufacturer's instructions state to immerse the dipstick in the urine sample, however on account of low sample volumes urine was instead pipetted directly onto the absorbent sides of the dipstick for each test kit until capillary action was observed in the device's test window. A positive result (i.e., above the cutoff) was indicated by the complete absence of a test line and the presence of a control line. Even a faint test line, accompanied by a control line, indicated a negative result. Note that this is the reverse of many common dipstick immunoassays for which the appearance of a test line instead indicates a positive result. Per the manufacturer's instructions, the results were interpreted after a minimum of five minutes of exposure to the urine, after which each dipstick was photographed for documentation. We recommend that future studies also collect the specific gravity of each urine sample to control for urine concentration and to facilitate future comparison with other studies [**79**] (see Discussion). Such a correction was not originally specified in the assay kit instructions and thus was not implemented in this preliminary work.

Due to constraints in assay kit availability, only 11 assays at a threshold of 500 mg/mL were available for use in the field, compared to >20 assays at a threshold of 300 ng/mL. We used 20 urine assays at the cutoff of 300 ng/mL to assay urine belonging to 9 females (two subadults) and 10 males (one subadult). These included three females in estrus and one adult male chimpanzee that was sampled twice over an interval of four days. A subset of eleven urine samples that passed the lower cutoff were then selected to be assayed at a threshold of 500 mg/mL a to evenly represent chimpanzees by sex, age and reproductive status, namely 4 adult males, 3 adult females, 3 additional adult females in estrus, and 1 subadult female.

To estimate possible ingestion of ethanol from fruits, we collected samples of *G. albida* opportunistically during periods when we followed chimpanzees. Ethanol concentrations were either assayed the same day of collection, or the fruits were frozen to arrest fermentation, and were later analyzed. We predominantly observed chimpanzees consuming fruits of *G. albida* within the tree canopy. However, chimpanzees were once briefly observed 'scrumping' this species from the ground upon first arrival at a new fruit crop [**80**]. We therefore prioritized collection of fruits that were directly observed to have fallen from trees, either spontaneously or en masse as a consequence of arboreal social displays and interactions among chimpanzees. Recently fallen fruits were also collected, as characterized by wet latex exudate from their stems. Fruits that were obviously aborted or unripe, fruits with bite marks, and fruits that were significantly damaged by ground impact were not collected. Given the opportunistic nature of the fruit collection, it was not possible to distinguish between fruits that fell naturally from those

that were rejected by chimpanzees; if chimpanzees are more likely to reject fruits with lower ethanol concentrations this would result in a more conservative estimate of ethanol values and vice versa. Fruit samples were binned by crop, as defined by the individual tree from which they originated; fruits from an indeterminate tree of origin were not collected. Chimpanzees were observed repeatedly returning to the same fruit crops many days apart and fruits from two such crops were opportunistically re-sampled.

Fruit-ethanol content was assayed using a metal oxide semiconductor (MOS) sensor method previously described in Maro *et al.* [**59**]. Briefly, we first prepared water-ethanol calibration standards by serially diluting 95% ethanol to concentrations of 0.4, 0.2, 0.1, and 0.05% weight per weight (i.e., % w/w). We then pipetted 3–4 g of each standard into a 50-mL centrifuge tube, and covered the top with non-porous plastic attached by rubber bands to form a septum. Fruit samples were prepared by homogenizing the pulp (i.e., excluding the exocarp and seeds) of the entire fruit, applying ~3–4 g of the resulting slurry evenly at the bottom of a 50 mL centrifuge tube, and similarly covering the tube with a septum. The headspace of samples was allowed to equilibrate for 30–40 minutes, after which the septum was pierced with a knife. The ethanol sensor was inserted to a point 1 cm above the sample, and was held in place until the sensor output voltage remained nominally constant for twenty seconds, at which moment it was recorded. Two ethanol sensors were used synchronously to accelerate lab work. Fruit samples were assayed alongside a corresponding set of calibration standards prepared the same day for each sensor.

We used R (v4.4.2) [**30**] to create a linear regression of instrument voltage on known ethanol concentrations for each calibration series (using the function 'lm'), which was then used to estimate fruit-ethanol concentrations (using the function 'predict'). Because the raw voltage output of the MOS instrument has a non-linear relationship to pre-specified ethanol values, we first log-transformed the known ethanol concentration values for each calibration series to obtain a linear regression. Predicted fruit-ethanol values resulting from each linear model were then inverse log-transformed using the exponential function. Thus, each set of fruits analyzed on a given day using a given instrument was paired with a fresh series of water-ethanol calibration standards, resulting in a total of ten linear regressions. All regressions had an adjusted R^2 values > 0.94, p-values < 0.02, and a mean absolute error averaging 0.016% (maximum of 0.026%).

Chimpanzees were observed consuming the fruits of *G. albida* crops in two distinct ways, predominantly using their incisors to scrape out the sweet fleshy mesocarp while also consuming the sweet fleshy endocarp, but occasionally only consuming the endocarp and discarding the mesocarp. We therefore compared ethanol concentrations between these tissues by dissecting out and assaying the mesocarp and endocarp separately for a subset of fruit samples. The mesocarp is distinct and easily peeled away from the endocarp (for illustrative photographs, see **Supplementary figure S1**, **2.5 Appendix**). The endocarp encloses 5–6 individual seeds, each situated within a mucilaginous membrane, which were removed by cutting away from and along a seed's edge with a knife, and then scraping the funiculus off from the seed with the back of the knife.

2.3 Results

Ethyl glucuronide concentrations were determined for 20 urine samples at a cutoff of 300 ng/mL, with urine from 17 chimpanzees exceeding this value (**Figure 2.1**). Eleven urine samples

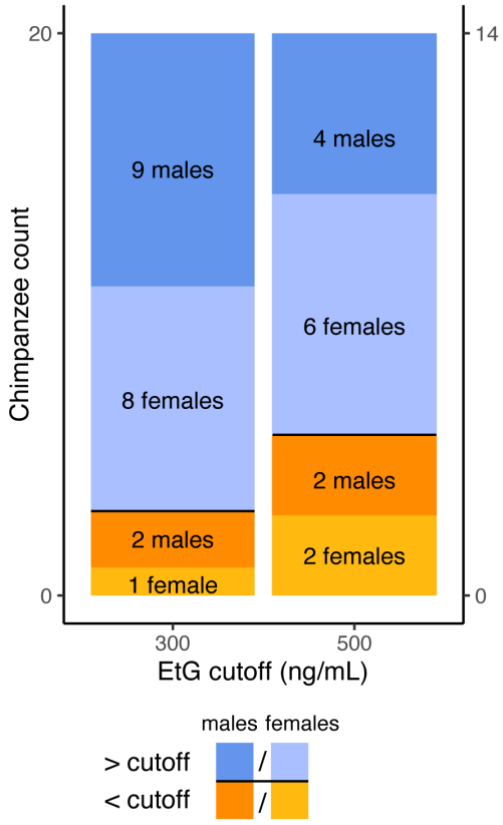


Figure 2.1: Results of monoclonal antibody-based enzyme immunoassays for 20 chimpanzee samples tested at a cutoff of 300 ng/mL, and a subset of 11 samples at a cutoff of 500 ng/mL. The stacked bar plots are subdivided by counts of positive test results above (blue) and negative test results below (orange) each respective cutoff. Each cutoff result is further differentiated by males (darker shade) and females (lighter shade). Three tests below the 300 ng/mL cutoff in the left-hand plot were also inferred to test negative at the 500 ng/mL cutoff, and were therefore included in the right-hand plot for a total sample size of 14.

that had passed the first cutoff were then sampled at the higher threshold of 500 ng/mL, with only one subadult female chimpanzee failing to surpass this higher cutoff (falling between 300 and 500 ng/mL), along with the three that did not pass the first cutoff (see **Figure 2.1**; **Supplementary table S2.1**).

Fruits were collected from nine *G. albida* trees over the course of two weeks overlapping the timing of urine sample collection, and from sites spanning the geographical ranges of both chimpanzee communities. Ethanol concentrations for pulp of these fruits averaged 0.09% (± 0.01 SE) (n=88), with values ranging 0.01–0.40%. Individual crop averages ranged from 0.03% to 0.17% ethanol (**Figure 2.2**). We found no significant difference in ethanol concentration between mesocarp and endocarp tissues (paired Mann-Whitney U test, $V = 19$, $p = 0.469$; **Supplementary figure S2.2**).

2.4 Discussion

EtG concentrations within urine samples of ten individual chimpanzees exceeded the threshold of 500 ng/mL, a level which is sufficiently high in humans as to rule out unintentional alcohol consumption (**Figure 2.1**; see [**63**,**67**–**69**]). Together with estimates of fruit-derived ethanol ingestion in the field, these results indicate a physiologically substantial intake of ethanol by chimpanzees during a mast fruiting event of *Gambeya albida* (**Figure 2.2**). Because urine samples were not assayed at higher thresholds, these results likely indicate a lower bound for ethanol exposure.

Chimpanzees obtain ethanol exclusively from fruit pulp, and feeding represents nearly half of their daily activity budget [**81**]. By contrast, studies of ethyl glucuronide kinetics in human urine typically provide liquid ethanol in acute doses, and over time spans ranging from minutes to an hour (e.g., [**68**,**82**–**84**]). These distinctions are important for two key reasons. Firstly, EtG accumulation in both human and rodent blood closely tracks the timing and amount of ethanol intake [**82**,**85**]. By contrast, slow rates of fruit ingestion by chimpanzees may elicit urinary EtG accumulation more slowly over time as compared to those for humans. Secondly, humans (and likely chimpanzees) produce alcohol dehydrogenase enzymes within the stomach during the first-pass gastric metabolism of ethanol, as indicated by significantly lower ethanol concentrations reaching the human duodenum relative to those within the stomach [**86**]. As a consequence, reduction in the rate of gastric emptying significantly increases the alcohol elimination rate, and also reduces and delays the amplitude and onset, respectively, of peak serum ethanol [**87**]. Also in humans, food consumption one hour prior to intravenous injection of ethanol significantly increases the intravenous elimination rate of alcohol, implicating additional elimination mechanisms such as increased liver blood flow, stimulation of alcohol enzyme activity, and increased rates of NADH reoxidation during alcohol metabolism [**88**]. These physiological effects, if present in chimpanzees, would suggest that higher rates of ethanol ingestion are required to surpass the same EtG thresholds indicated by the test methodology used here, which is calibrated internally for human exposure. Moreover, since chimpanzees are ripe fruit specialists [**25**]**,** their capacity to metabolize ethanol, as chronically ingested via fermented fruit, may thus have evolved to be correspondingly faster.

Correlations between EtG levels in urine with rates of alcohol consumption by humans can nonetheless be used to initially approximate overall intake by chimpanzees. Albermann *et al.* [**68**] assayed urinary EtG of human subjects 24 hours after they had consumed 1–2 drinks

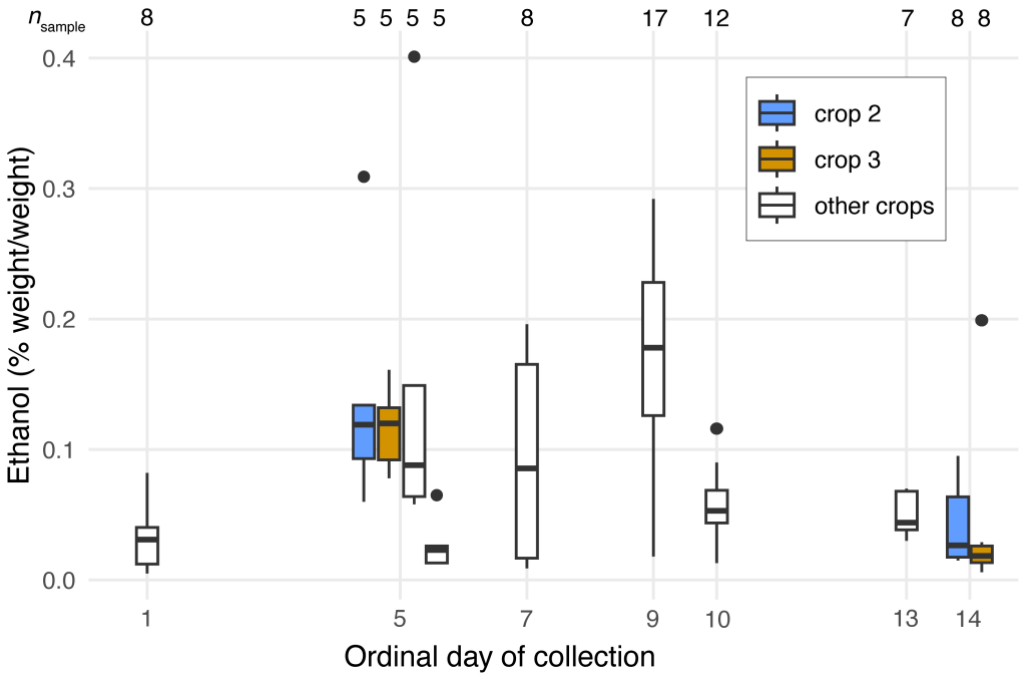


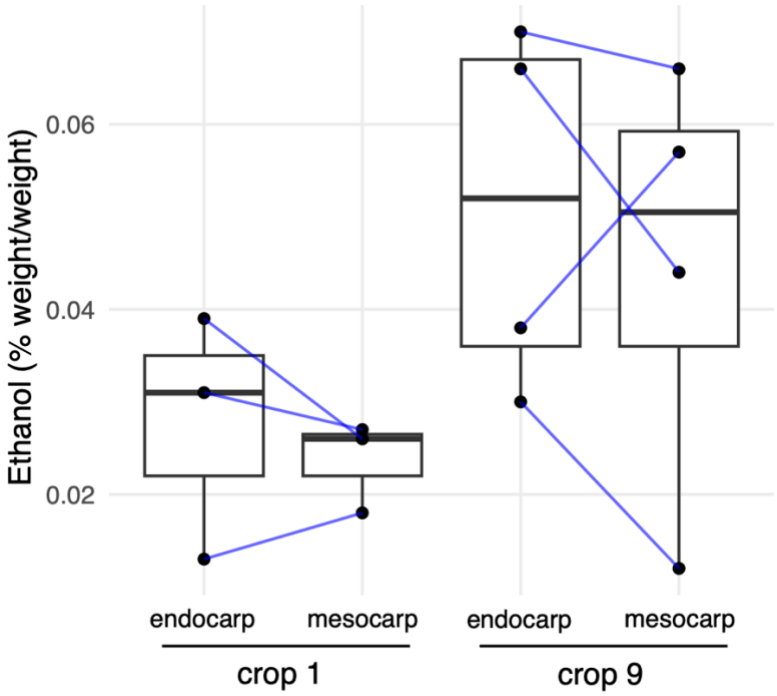
Figure 2.2: Average pulp-ethanol concentrations for nine *Gambeya albida* fruit crops collected over two weeks (August 9–22, 2025). Two crops (#'s 2 and 3) were each collected twice (i.e., on day 5 and day 14). Box plots follow standard interquartile range notation. Sample size (n_{sample}) for each studied fruit crop is indicated at the top of the chart.

(corresponding to an ethanol dosage ranging from 0.12–0.32 g/kg); only two of twelve subjects exceeded an EtG cutoff of 500 ng/mL. Chimpanzees at Ngogo, by contrast, obtain via frugivory an estimated daily ethanol dosage of 0.34–0.38 g/kg [**59**], consistent with a larger fraction of individual samples exceeding the aforementioned EtG cutoff. One interpretational caveat is that, in general, decreased water intake may lead to increased urine concentrations and correspondingly inflated concentrations of EtG. However, EtG concentrations can be normalized to levels of urine creatinine, a metabolite deriving from muscle activity [**89**]. For example, none of the aforementioned subjects in Albermann *et al.* [**68**] exceeded the 500 ng/mL cutoff after creatinine normalization. Although chimpanzees do not typically consume free water when water-rich fruits are available [**90**], variable urine concentration among individuals is a potentially confounding variable. The specific gravity of urine is closely correlated to its creatinine level [**79**], and can be easily measured and used to normalize EtG estimates among samples. Moreover, assay detection cutoffs can be extended to higher EtG concentrations by diluting urine with saline [**71**], and then normalizing by the ensuing specific gravity.

In sum, these results indicate substantial ingestion and assimilation of dietary ethanol by both male and female chimpanzees during the mast fruiting event of a preferred and calorically dense species [**74**,**75**]. Methodologically, natural ethanol consumption via frugivory can now be inferred from mammalian urine samples by measuring the direct metabolite ethyl glucuronide using field-portable immunoassays. Substantial presence of EtG in chimpanzee urine buttresses comparable findings of Campbell *et al.* [**40**] for spider monkeys, and further supports the "drunken monkey" hypothesis that the widespread occurrence of ethanol within tropical fruit is not aversive to frugivores at its naturally low concentrations [**7**]. Future studies using this approach also have the potential to assess effects of dietary ethanol on the physiology and behavior of focal animals over time (e.g., effects on the initiation of boundary patrols and intergroup aggression [**91**] or on the timing of estrus in chimpanzees [**76**,**77**]), thus potentially revealing new drivers of frugivore foraging ecology.

2.5 Appendix

Supplementary figure S2.1: Photographs of the African star apple *Gambeya albida*. (**A**) An example of a fruit for which the mesocarp was consumed by chimpanzees. (**B**) An example of fruits for which the mesocarp was not consumed by chimpanzees. (**C**) The endocarp (and the seeds it encloses, left hand side) easily separates from the darker orange mesocarp (right hand side).



Supplementary figure S2.2. Comparison of ethanol concentrations between mesocarp and endocarp tissues belonging to two crops of *Gambeya albida***.** Chimpanzees consuming crop 1were observed to eat both the endocarp and mesocarp, whereas chimpanzees consuming crop 9 were observed to eat only the endocarp. The blue lines indicate which fruit the endocarp and mesocarp both came from. Ethanol concentrations were not significantly different between the mesocarp and endocarp for crop 1 (paired Mann-Whitney *U* test, $V = 4$, $p = 0.75$; n=3), for crop 9 (paired Mann-Whitney *U* test, $V = 7$, $p = 0.625$; n=4), or for both crops combined (paired Mann-Whitney *U* test, $V = 19$, $p = 0.469$).

Supplementary table S2.1. Ethyl glucuronide immunoassay results. Results of monoclonal antibody-based enzyme immunoassays of 19 chimpanzees. Note one chimpanzee named Wes was tested twice four days apart at a threshold of 300 ng/mL, and passed the first test prior to failing the second one. A (*) symbol indicates negative test results at the 500 ng/mL cutoff that were inferred from failure at the 300 ng/mL test. Chimpanzees with a (†) symbol were in estrus during the time of collection.

chimpanzee	age	age class	sex	date	Ngogo community	300 (EtG ng/mL)	500 (EtG ng/mL)
Zawinul	14	adult	M	15-Aug	Central	Y	Y
Miles	46	adult	M	15-Aug	Central	Y	Y
Evans	29	adult	M	15-Aug	Central	Y	Y
Francoise	14	adult	F	18-Aug	Western	Y	Y
Binoche	34	adult	F	18-Aug	Western	Y	Y
Frika†	14	adult	F	19-Aug	Western	Y	Y
Sabin†	28	adult	F	20-Aug	Western	Y	Y
Damien	19	adult	M	20-Aug	Western	Y	Y
Ellen	20	adult	F	21-Aug	Western	Y	Y
Lombard	11.4	subadult	F	21-Aug	Western	Y	Y
Vigilante	10	subadult	F	21-Aug	Western	Y	N
Herzog	10.5	subadult	M	17-Aug	Central	N	N*
Justine†	17	adult	F	21-Aug	Western	N	N*
Wes	28	adult	M	22-Aug	Western	N	N*
Bobby Wayne	15.2	adult	M	17-Aug	Central	Y	
Wes	28	adult	M	18-Aug	Western	Y	
Larson	17	adult	M	18-Aug	Western	Y	
Wayne	29	adult	M	19-Aug	Western	Y	
Leigh	24	adult	F	20-Aug	Western	Y	
Murray	21	adult	M	21-Aug	Western	Y	

* Negative test results at the 500 ng/mL cutoff were inferred from failure at the 300 ng/mL test

† Chimpanzees were in estrous during the time of collection.

Chapter 3

Low-level ethanol is widespread within floral nectar

3.0 Abstract

Floral nectar is a major part of the diet of many animals, which can consume daily nectar volumes equivalent to their body mass. Saccharide-rich nectar may undergo fermentation after becoming inoculated with microbes, yet empirical studies surveying nectar-alcohol concentrations are sparse. We used an enzymatic assay to estimate ethanol concentrations for 147 samples of nectar from 29 species of flowering plants at a botanical garden. Ethanol was detected within 48% of all nectar samples, with at least one ethanolic sample for 26 species. Ethanol-positive samples contained concentrations averaging 0.016% (weight/weight) by species, with a maximum species average of 0.032% and a maximum sample concentration of 0.056%. The resulting estimated body mass-adjusted daily ethanol dosage for associated pollinators is considerable, roughly comparable to a single alcoholic beverage consumed by a 70 kg human over the course of a day. Additional field and metabolic studies are needed to determine to what extent such ethanol concentrations are either physiologically or ecologically relevant for animal consumers. Overall, our results indicate that exposure to low levels of ethanol amongst nectarivores is likely to be widespread.

3.1 Introduction

Animals routinely encounter potentially hazardous substances in their diets that require detoxification and that affect their food preferences [**92**,**93**]. Over evolutionary time, such foraging constraints can result in coevolutionary outcomes such as arms races [**94,95**] and compound sequestration [**96**], which can influence ecosystems on multiple trophic levels [**97**]. For example, many flowering plants produce a variety of secondary compounds to protect them from herbivory, such as caffeine and nicotine, which are expressed in high concentrations throughout various tissues and within floral nectar, which additionally affects pollinator preferences [**98**–**101**]. Nectar microbial composition can also be influenced by secondary compounds, and conversely some microbes are able to reduce the concentration of secondary compounds and lower deterrence to pollinators [**102**]. Understanding how animals deal with naturally occurring toxic substances can advance our understanding of their feeding ecology, and the broader eco-evolutionary context of these interactions.

The dietary and foraging ecology of any animal that consumes high volumes of fruit or nectar is expected to include exposure to alcohol, as foods containing simple sugars naturally undergo fermentation after being colonized by yeasts [**103**,**104**]. Metabolism of alcohols (mostly ethanol) from fermented sugars may be challenging to animals lacking corresponding adaptations, and ethanol at high levels can be toxic. The ability to metabolize alcohol can also provide caloric and other physiological benefits at low but naturally occurring levels [**39,105**–**108**]. Such a dose-dependent interaction, with positive effects from consumption of small quantities of the substance, and negative outcomes from no dose and high doses, is an example of hormesis [**39**]. A hormetic relationship to alcohol has been previously demonstrated in fruit

flies (*Drosophila melanogaster*) [**105**]. In humans, moderate drinkers have similarly been shown to exhibit 16% lower all-cause mortality relative to abstainers due primarily to cardiovascular effects [**106**–**108**], however these results have been widely disputed [**109**–**111**]. Nevertheless, naturally occurring alcoholic fermentation likely affects the feeding ecology of frugivores such as chimpanzees [**59**] and our own human ancestors [**6**], as well as monkeys [**40**] and bats [**43**]. Likewise, some nectarivorous mammals such as pen-tailed tree shrews (*Ptilocercus lowii*) and slow lorises (*Nycticebus coucang*) are exposed to dietary ethanol in the wild [**41**], and slow lorises and aye-ayes (*Daubentonia madagascariensis*) in captivity have been shown to significantly prefer sucrose solutions with 2–4% and 3–5% ethanol concentrations, respectively, over 0–1% concentrations [**45**]. The nectarivorous Anna's hummingbird (*Calypte anna*) and a variety of other bird species were shown to contain an ethanol metabolite sequestered within their feathers, at concentrations suggestive of chronic alcohol consumption in human hair [**66**]. Nectarivorous pollinators such as hummingbirds (Trochilidae), sunbirds (Nectariniidae), and insects (e.g., honeybees, *Apis mellifera*) might also be routinely exposed to ethanol through the fermentation of nectar by microbes. Although ethanol has been detected in surveys of fruit, ethanol levels within nectar are largely unknown (for a general review of the evolutionary ecology of ethanol, see [**8,112,113**]). The goal of this study was to survey nectar from a large variety of plants to determine to what extent ethanol can be found within this nutritional resource. Nectarivory has evolved at least 11 times across Aves [**114**] and at least 10 times across Insecta [**115**], and estimation of the ethanol dosage associated with chronic natural exposure can inform a more complete understanding of the adaptive hurdles involved in such a dietary transition.

The potential for ethanol to occur in nectar is tied to the occurrence and ecology of fermentative species of microbes. The microbial ecology and community assembly of yeasts and bacteria within floral nectar has been studied extensively [**116**]. Early work found mutualisms between nectar yeasts and bumblebees [**117**]. Subsequent studies showed that sugar-nectar concentrations change in proportion to yeast cell density [**118**], and that yeasts are transferred by insect pollinator visitation [**119**]. Yeast-insect pollinator relationships have been described as a 'dispersal–encounter' hypothesis wherein insects vector yeasts to sugar-rich nectaries in exchange for an honest cue from the yeasts about the condition of the sugars via olfaction of resulting fermentation compounds such as ethanol [**120**,**121**]. Analysis of volatile compounds produced by the common and widespread nectar yeast specialist *Metschnikowia reukaufii* and the more generalist yeast *Aureobasidium pullulans* showed that ethanol and its derived compounds exhibit by far the largest signal [**122**,**123**]. Despite such mounting evidence of a likely ecological role of ethanolic fermentation across the worldwide diversity of flowering angiosperms, ethanol concentrations themselves have never been systematically documented within floral nectar. The sole exception is nectar of the tropical bertam palm (*Eugeissona tristis*), which averaged an ethanol concentration of 0.8% and was found to contain brewer's yeast (*Saccharomyces cerevisiae*) among a plethora of other fermentative microbes [**41**]. The bertam palm's inflorescence evolved to attract mammalian pollinators, among which is the pen-tailed treeshrew (*Ptilocercus lowii*) which was estimated to ingest a dosage of 1.4 grams of pure ethanol per kilogram of body mass per day because of nectar consumption [**41**]. Estimating ethanol concentrations commonly found within floral nectar more broadly is a first step in assessing the likelihood of the evolution of corresponding olfactory and gustatory responses within pollinator lineages. Estimation of the daily ingested volume of ethanol by various

pollinators may further indicate the extent to which dietary ethanol imposes physiological tradeoffs on nectarivorous taxa.

Surveys of the presence of microbes within floral nectar may indicate the extent to which we can expect to find fermentatively derived ethanol. At least half of all described yeast species are capable of ethanol production under fermentation [**124**,**125**], whereas bacteria produce a much wider variety of fermentation end products at lower concentrations than do yeasts, and bacteria typically do not produce significant amounts of ethanol [**122**,**126**]. We can therefore expect ethanol to be found within yeast-inhabited nectaries but not those inhabited solely by bacteria. Limited surveys in two Mediterranean and one subtropical climate indicate that the occurrence of microbes within floral nectar is widespread, with yeasts found in 20–43% of samples, bacteria within 17–49% of samples, and the co-occurrence of members of both groups in 14–17% of samples [**127**–**129**]. In tropical regions, fungal diversity within floral nectar is characterized by many common species with low dominance composed of roughly equal parts yeast (Ascomycota; 48%) and filamentous fungi (Basidiomycota; 52%) [**130**]. In temperate regions, nectars typically contain one of a few specialist yeast species, most prominently members of the genus *Metschnikowia* or otherwise one of many transient filamentous fungal species [**131**]. Thus, yeasts that could ferment nectar appear to be common in many habitats.

We hypothesized that there could be a relationship between nectar ethanol concentrations, nectar sugar concentrations, and the primary pollinator associated with each species. Pollinator preference for specific floral traits, i.e., a pollinator syndrome, is thought to be a major driver of angiosperm evolution [**132**,**133**]. Hummingbirds and sunbirds are broadly associated with flower species containing lower nectar sugar concentrations than those of insects (i.e., 25.4%, 23.4%, and 41.6%, respectively) [**134**]. Pollinator floral preference has been shown to correlate with microbial composition and diversity in nectar for hummingbirds (*Calypte anna*) [**135**] and honeybees (*Apis mellifera)* [**136**], both of which consumed ~30% less nectar from synthetic flowers inoculated with commonly occurring bacteria than uninoculated control nectar and nectar containing *M. reukaufii*.

Ethanol itself may affect pollinator dietary preferences. Ethanol concentrations increased over time within hummingbird feeders containing 20% sucrose and also increased with weekly temperatures, peaking at a concentration of 0.05% [**137**]. However, Anna's hummingbirds were just as likely to consume artificial nectar with 1% ethanol as with no ethanol [**137**], making it unlikely that the peak 0.05% concentration found in feeders would affect preference. Honeybees on the other hand have been found to significantly prefer 1.25 and 2.5% ethanol concentrations within artificial nectar relative to 0% controls or to higher concentrations of 5% ethanol [**138**]. Honeybees, like humans, voluntarily consume alcohol in concentrations as high as 20% [**139**] and chronic alcohol consumption as high as 80% alcohol has been shown to have no effect on the mortality or behavior of Oriental hornets (*Vespa orientalis*) [**140**]. Moreover, the survivorship, flight endurance, body mass and lipid content of honeybees were unaffected by 1% chronic ethanol consumption [**141**]. Nonetheless, even low concentrations of nectar ethanol may be ecologically informative for foraging decisions and physiologically significant given the large nectar volumes energetically required by pollinators relative to their body mass [**142**–**146**].

We hypothesized that ethanol would be found in nectar based on the widespread presence of fermentative microbes, the ability of pollinators to detect ethanol, and observations of ethanol in fruit which is similarly high in sugar content [**7**,**59**]. We tested this hypothesis by quantifying ethanol concentrations within floral nectar in a broad range of flower species in a common

garden located in a Mediterranean climate. We then evaluated to what extent nectar ethanol concentrations were correlated to sugar concentrations, known pollinators of the species, and their phylogeny. Finally, we used published values of daily caloric intake and body size to estimate the average ethanol dosage ingested by various nectarivores.

3.2 Materials and Methods

3.2.1 Study site & sample collection

Nectar samples were opportunistically collected from the University of California, Berkeley Botanical Garden in Berkeley, CA, USA (N 37°52'30.4", W 122°14'18.6"), twice in April and once in July and in August of 2022. In addition, two subsets of samples belonging to the local native hummingbird-pollinated species *Malva assurgentiflora* and *Epilobium canum* were opportunistically collected at a private residence 13 km northwest in Hercules, CA, USA (October–November of 2022 and April–May 2023). Nectar was extracted from flowers using a 5 μL microcapillary pipet (71900-5, Kimble Chase, Vineland, NJ, USA), which was then sealed by pressing the pipet ends into sigillum wax sealant (51601, Globe Scientific, Mahwah, NJ, USA). The pipets were labeled using tape, sealed inside of a modified 50 mL centrifuge tube and kept on ice until storage in a -20°C freezer on campus. The samples were additionally sealed for long-term storage inside of a thick polystyrene box layered inside of a cardboard box, intended to prevent air movement and minimize evaporation. The species identity of plants sampled at the Botanical Garden were either clearly marked by identifying placards on site or identified with the assistance of garden staff. Two species sampled in Hercules were identified by the species name of the plant when it was purchased. Each species was sampled with the goal of collecting at least three data points. Individual flowers were not re-sampled. Nectar samples were inspected within their microcapillaries, and any obvious visible color impurities were noted. The time of day was recorded at the collection of each sample. Temperature and humidity associated with samples collected at the Botanical Garden were ascribed using data collected every 15 minutes at the neighboring Lawrence Berkeley National Laboratory weather station, downloaded from MesoWest [**147**]. Temperature and humidity associated with samples collected at the alternate site in Hercules, CA were recorded using a stainless steel temperature probe (TMP-BTA, Vernier Software and Technology, Beaverton, OR, USA) and a relative humidity sensor (RH-BTA, Vernier Software and Technology) connected to a touchscreen interface (LabQuest 2, Vernier Software and Technology).

3.2.2 Nectar ethanol assay, calibration, and validation

Ethanol concentrations were assayed following the suggested protocol of a commercial kit utilizing alcohol dehydrogenase catalyzed oxidation of ethanol (ECET-100, BioAssay Systems, Hayward, CA, USA). The kit was developed for quantitative determination of ethanol with a detection limit of 0.0008 % (volume/volume) ethanol [**148**]. Each nectar sample was kept frozen until the assay materials were all prepared. The nectar was extracted from its sealed microcapillary tube by filing down the waxed ends, snapping them off and transferring the contents into a 0.5 mL microcentrifuge tube using a microinjector. All nectar samples were then diluted with purified water (1:9) during analysis to ensure sufficient assay volume. Nectar samples and one or more series of calibration standards were assayed inside a black flat-bottom

96-well microplate (Greiner Bio-One, Monroe, North Carolina, USA), with resulting absorbance values collected on a spectrophotometer (Spectramax M2, Molecular Devices Corporation, Sunnyvale, CA, USA). Samples were measured in four sets (referred to as plates 1–4).

Calibration standards were initially composed of a four-point serial dilution sequence (0, 0.03, 0.06, 0.10 % volume/volume; alongside n=8 nectar samples), as suggested by the manufacturer's protocol [**148**]. For the second series of calibration standards (plate 2), we added a smaller calibration standard of 0.01% and dropped the large 0.10% value, because assays of plate 1 revealed that nectar contained low concentrations of ethanol. To facilitate subsequent assays incorporating sugars (which we measured in units of mass), all subsequent percentage values reported for ethanol standards reported here are converted to units of weight/weight (w/w). The final two calibration standard series (plates 3–4) incorporated values of 0.10%, 0.06%, 0.03%, 0.01%, 0.005% and 0.001% ethanol (w/w). Once calibration standards and diluted samples were prepared, we added freshly prepared assay kit reagents, incubated the samples for 30 minutes, and then added the stop reagent as specified in the protocol [**148**].

To determine if nectar sugar concentrations covary with ethanol content, we estimated the undiluted saccharide concentration of each nectar sample using a handheld Brix refractometer (Westover RHB-32ATC, Laxco, Inc., North Creek, WA, USA), as is typical in studies of nectar characteristics (e.g., [**149**,**150**]). For simplicity, values of °Brix will be referred to as sugar concentrations throughout the text. Since nectar is naturally very sweet, with typical concentrations around 20% (and ranging in excess of 40%), we experimented using variable sugar concentrations within the calibration standards to see if such variation affects the assay kit's performance. For all assay plates excluding the first, we prepared calibration series with ethanol as described above, but containing 0%, 2% and 4% sucrose (w/w), (given that all nectar samples were diluted 10-fold). We then conducted an analysis of covariance (ANCOVA) using the 'anova_test' function in the 'rstatix' package in R (v4.4.2) [**30**], with absorbance as the dependent variable, ethanol concentrations as a continuous independent variable, and sugar concentrations (0, 2 or 4%) as a categorical independent variable. The results of this ANCOVA showed a significant correlation between absorbance values and ethanol concentrations (p<0.001), but sucrose concentrations did not significantly contribute to the prediction of variance in absorbance values ($F_{2,48}$=2.4, p=0.102), even if each plate was analyzed separately ($F_{2,8}$=2.2, p=0.179, $F_{2,14}$=0.9, p=0.426, and $F_{2,14}$=3.2, p=0.072, corresponding to plates 2–4 in **Supplementary figures S3.1 and S3.2**).

Since sucrose is often not the most common form of sugar naturally found in nectar, for plate 2 we also assessed whether calibrating with fructose or glucose would affect measurements of ethanol in standardized solutions. Again, an ANCOVA model was used with absorbance as the dependent variable, ethanol concentrations as a continuous independent variable, and sugar type (sucrose, glucose, or fructose) as a categorical independent variable. These different types of sugars (all assayed at a concentration of 2%) did not predict absorbance values of calibration standards (ANCOVA, $F_{2,8}$=0.1, p=0.868). Given the lack of a consistently significant correlation between sugar concentration and absorbance, we pooled calibration standards across all sugar concentrations for each of these plates when estimating the relationship between absorbance and ethanol in the standards.

We then evaluated how to best model the relationship between ethanol calibration standards and their resulting absorbance values. To fit and evaluate linear regressions for ethanol assay calibration, we used the 'lm' function in the 'stats' package and visualized the regressions

using the 'ggtrendline' function in the 'ggtrendline' package. Both linear and power regression models had similarly high adjusted R^2 values for all plates (all R^2>0.95). At lower concentrations where most of our samples were clustered, however, power regression models better fit the calibration data than linear models (**Supplementary figure S3.1**) and linear models forced through the intercept (**Supplementary figure S3.2**). Ethanol concentrations were therefore predicted using power regression models for 213 samples of nectar. Of these, 162 samples were described as 'clear' and 51 samples were noted as having various shades of yellow, orange, tan, brown, or 'cloudy white' coloration prior to dilution during sample preparation. These latter samples yielded significantly higher ethanol estimates than did the 162 clear samples (0.031% vs 0.010%; Student's t-test, p<0.001), likely due to higher absorption values as a result of color impurities, and were excluded from further analysis. We only considered species with at least three samples for subsequent analyses, which resulted in the exclusion of 15 additional samples from species that did not meet this threshold. A total of 17 samples of the remaining 147 samples yielded absorbance values lower than the zero-ethanol samples, resulting in negative ethanol values (averaging -0.002%), which were rounded up to 0%. As a result of the dilution of nectar samples 1:9 with water, the limit of detection of ethanol within the nectar samples effectively rose to 0.008%, and a total of 51 samples resulting in positive ethanol values below this cutoff were relabeled as zero.

3.2.3 Identification of pollinators

We used a literature search to determine which pollinators are associated with each plant species that we sampled for nectar. We assessed which pollinator species are associated with each species of flower within its native range, searching for "*genus species* pollinator" in Google Scholar and in the Web of Science. This method alone was sufficient for all but four species. One species, *Pitcairnia* sp., was only identified to genus in the Botanical Garden records, and was ascribed a pollinator class based on the flower's morphological clustering with other members of the genus for which pollinators were known. The species *xAmarcrinum memoria-corsii* is an artificial hybrid, and we ascribed its pollinator class based on that of the crossed species. The species *Opuntia elatior* and *Rhododendron glanduliferum* are both relatively poorly studied members of large genera. We described *O. elatior* as insect-pollinated (entomophilous) based on the evidence that bees and other insects pollinate most of the members of the genus *Opuntia*, and because *O. elatior* does not possess the distinct secondary morphological adaptations present in explicitly hummingbird-pollinated species [**151**]. We treated *R. glanduliferum* as generally bird-pollinated (ornithophilous) based on its phylogenetic proximity to nine other members in the subgenus *Hymenanthes*, all native to the same geographic region and which are bird-pollinated [**152**]. We initially categorized the identity of the pollinator as specifically as possible, and then grouped pollinators into more general categories to obtain sufficient sample sizes for statistical tests. We divided species of flowers into four groups: entomophily (insect-pollinated; n=6), trochilophily (hummingbird-pollinated; n=12), nectariniophily (sunbird-pollinated; n=6), and "other" (n=5, including three bat-pollinated and two generally bird-pollinated species).

3.2.4 Statistical analyses

We used Blomberg's *K* to assess whether there is a phylogenetic signal associated with floral ethanol concentrations or with floral sugar concentrations (as indicated by °Brix) [**153**]. Blomberg's *K* values less than one indicate that related taxa are less similar than expected under Brownian motion evolution, whereas *K* values greater than one indicate that related taxa resemble each other more than expected under Brownian motion evolution [**153**,**154**]. The *p*-value associated with Blomberg's *K* indicates the proportion of time randomized trait data across the phylogeny results in a higher value of *K* [**154**]. Blomberg's *K* values were calculated from averaged species data using the 'phylosig' function in the 'phytools' package in R [**30**].

We assessed correlations of ethanol concentrations with sugar concentrations and known floral pollinator group, while controlling for species identity and phylogenetic effects, by creating a series of phylogenetic generalized linear mixed models (PGLMMs) using the 'pglmm' function in the 'phyr' package [**31**] in R [**30**]. Additional coefficient of determination estimates associated with each model were made using the 'R2' function in the 'rr2' package [**33**]. A species phylogeny was obtained using the 'phylo.maker' function (under 'scenario 2') of the 'V.PhyloMaker2' package in R [**32**]. Fixed variables used in the models included sugar concentrations, pollinator group, ambient temperature, relative humidity and the relative time of day. To better understand the phylogenetic effects of the models we subdivided the species into five clades, each with a sample size $n \geq 4$ and diverging ~118 million years ago, which resulted in a clade belonging to asterids (n=7 species), the Cactaceae family (n=4), and malvids (n=6) among the eudicots, and the order Asparagales (n=9), and the family Bromeliaceae (n=4) among the monocots; these clade identities were also used as a fixed effect. Species were similarly grouped by monocot (n=12) and eudicot (n=17) as an additional fixed effect. At most only two fixed effects were combined in a single model at a time and categorical fixed effects were never combined. Phylogenetic data and species identity were included as "random" variables, alongside the residual variance. A series of PGLMMs were created using the maximum likelihood (ML) estimation method to compare the effects of each of the fixed variables [**155**]. A PGLMM was also created without phylogenetic effects, to compare only the effect of species identity, using the same R function. Models were ranked using the R^2 value associated with likelihood estimation (R^2_{lik}), as well as the Akaike Information Criterion [**33**]. As a point of comparison to ethanol, we additionally created a series of PGLMMs to model sugar concentrations as a dependent variable against the same floral traits.

3.2.5 Dosage estimates

Existing literature describing the average daily sugar intake and body mass of various pollinators were used to estimate the daily exposure of those animals to ethanol levels found in this study. For example, wild caught Anna's hummingbirds (*Calypte anna*) require the equivalent of 1.98 g of sucrose per day to maintain their daily energy budget [**142**]. Floral nectar is a poor source of nitrogen, and hummingbirds and sunbirds are known to forage for insects [**156**]. However, in one study three Anna's hummingbirds were found to consume a similar amount of artificial nectar from feeders alone (ranging 10–14g of 22% sucrose artificial nectar) as from feeders with fruit flies also available (ranging 9–16 g of 22% sucrose artificial nectar) over a period of 10 days [**157**], suggesting that the nutritional value of insects as a nitrogen supplement is calorically negligible for Anna's hummingbirds. Likewise, pollen is generally thought to provide for the nitrogen requirements of honeybees whereas the carbohydrates for energy demands come from

honey [**158**]. For simplicity we assume all calories are derived from nectar. An Anna's hummingbird feeding exclusively on 20% sucrose nectar (w/w) would therefore need to ingest 9.9 g of nectar daily. This volume of nectar is ~2.21 times the average body mass of 4.48 g [**142**]. A hummingbird ingesting this volume at the average concentration of ethanol found in hummingbird feeders (0.013%) [**137**] would consume 0.0013 g of pure ethanol, resulting in a daily dosage of 0.30 g ethanol per kilogram body mass per day (i.e., g/kg/day). For several pollinator species with available daily energetics and body mass data, we similarly calculated dosage values using sugar and ethanol concentrations obtained from nectar in this study. For honeybees, we used a standard conversion factor of 21.2 kilojoules per liter of metabolic CO_2 produced, and a conversion of 17 kilojoules per 1 gram of sucrose [**158**].

3.3 Results

3.3.1 Ethanol concentrations

Ethanol concentrations (w/w) were determined for a total of 147 samples of floral nectar from 29 species. Among all samples, 48% contained detectable concentrations of ethanol, and least one sample from 26 out of 29 plant species contained ethanol concentrations above the kit's limit of detection. The average species-level (n=29) nectar ethanol concentration was 0.010%. The average species-level ethanol concentrations using only non-zero ethanol values (n=26 species) was 0.016% (w/w). The maximum species-average was 0.032%, and the maximum sample concentration was 0.056% (**Figure 3.1**).

Blomberg's K was 0.08 (p=0.045), indicating a weak phylogenetic signal of ethanol concentrations under Brownian motion evolution [**153**,**154**]. Species identity as the only random effect in a generalized linear mixed model (GLMM) resulted in an R^2_{lik} of 0.16 and the addition of phylogenetic effects as an additional random effect resulted in a lower (more parsimonious) Akaike's Information Criterion (AIC) score and further increased the model's R^2_{lik} value to 0.24 (phylogenetic generalized linear mixed model, PGLMM). Subdividing the species into five clades improved the AIC score of the model the most and increased the R^2_{lik} value to 0.29; this model indicated that species from the Cactaceae family had significantly higher ethanol concentrations compared to each of the remaining clades, which all had a negative effect size (all p<0.003) when compared to Cactaceae as the reference group in the model (PGLMM). Sugar concentrations were significantly correlated with an increase in ethanol when incorporated as a continuous variable in a PGLMM (R^2_{lik} =0.26, p=0.046) (**Figure 3.2**). Model fit was not improved by inclusion of pollinator group as a trait, nor by the designation of species as monocot/eudicot, nor by the ambient temperature, relative humidity or time of collection of the samples.

3.3.2 Sugar concentrations

Species-level nectar sugar concentrations, as indicated by °Brix, averaged 23% and ranged from 8–51%, with a maximum sample concentration of 68% (**Supplementary figure S3.3**). Blomberg's K was 0.13 (p=0.022), indicating a weak phylogenetic of sugar concentrations under Brownian motion evolution [**153**,**154**]. This was reflected in the PGLMM model which was unimproved by the addition of higher level phylogenetic effects. Species identity on its own

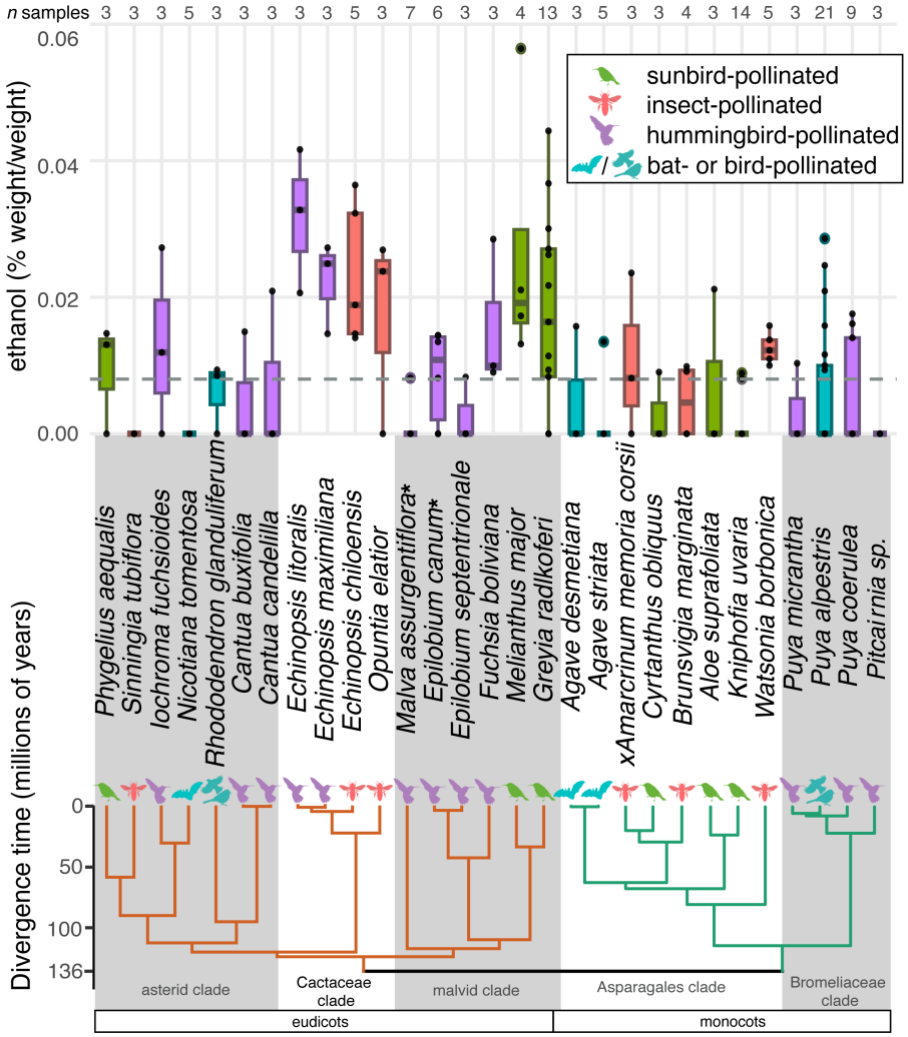


Figure 3.1: Ethanol concentrations of floral nectar for 29 angiosperm species, arranged according to phylogenetic relatedness [**32**]. All species were collected at the UC Botanical Garden except *Malva assurgentiflora* and *Epilobium canum*, which are marked with an asterisk (*).Green branches in the phylogeny refer to monocot species, and orange branches refer to eudicot species. Groups of species were classified into clades as indicated by gray alternating with white shading, as labeled at the bottom of the chart. Boxplots are color-coded by the expected pollinator for that plant species, with corresponding animal silhouettes (from PhyloPic) at the phylogeny tips: purple = hummingbird-pollinated (trochilophily), green = sunbird-pollinated (nectariniophily), salmon = insect-pollinated (entomophily), and cyan = "other" (including chiropterophily, ornithophily, and general zoophily). A grey dashed line demarcates the detection limit of the assay at 0.008%, below which values were rounded down to zero. Boxplots represent the 25th to 75th percentiles of the interquartile range (IRQ), a thick line color-coded line represents the median, and whiskers represent the minimum and maximum. The median, boxplot and whisker calculations exclude outliers, which are shown as points and represent ethanol values greater than the IRQ multiplied by 1.5, as added to the 75th percentile. Sample size (*n*) is indicated above each boxplot.

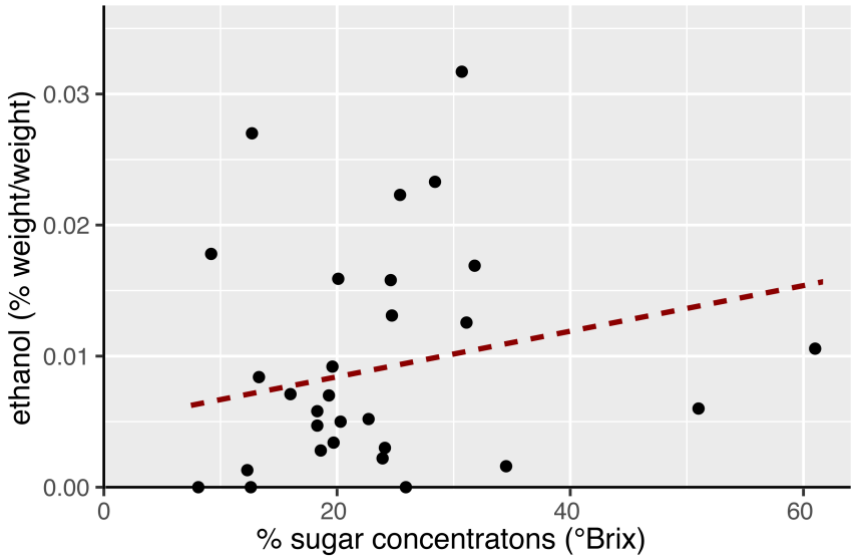


Figure 3.2. The relationship between sugar and ethanol concentrations in nectar. The phylogenetically-corrected correlation is displayed as a red dashed line (PGLMM, p=0.046). The model explaining variation in ethanol concentrations used sugar concentrations as the fixed trait and species identity and phylogenetic relationships as random effects.

predicted average nectar sugar concentrations with a R^2_{lik} of 0.57 (GLMM). Such a high R^2_{lik} is due to the low amount of variation in sugar concentrations within each species, making the species easy to predict based on sugar concentrations. However, predictable patterns within species do not mean that there are predictable patterns across species based on the phylogeny, which is reflected in the low levels of phylogenetic signal. The most parsimonious model (with the lowest AIC score among PGLMM models) also incorporated pollinator identity and ethanol concentrations as fixed traits which slightly increased the R^2_{lik} of the model to 0.60. In this model, sunbird-pollinated flowers contained significantly lower nectar sugar concentrations (average of 15.7%) than flowers pollinated by insects (30.5%; PGLMM, p=0.005), and nearly significantly lower than that of hummingbirds (23.1%; p=0.078) and "other" (24.8%; p=0.085) pollinator groups. Model fit was not improved by inclusion of the remaining fixed effects.

3.3.3 Ethanol dosage in hummingbirds, sunbirds, and honeybees

We located existing literature on daily energetic needs and average body mass for two hummingbird species, three sunbird species, and for honeybees, which we then used to estimate a hypothetical daily exposure to ethanol in units of grams per gram body mass per day (**Table 3.1**). For simplicity, we assumed that all daily energetic intake of these animals came from nectar alone, however hummingbirds and sunbirds eat some insects [**156**] and honeybees also consume pollen [**158**]. We also assumed no seasonal fluctuation and no foraging preference for or against ethanol. To calculate dosage, we used nectar-ethanol and nectar-sugar concentrations from the present study, corresponding to each specific pollinator group as associated with each flower. We estimated that Anna's hummingbirds (*Calypte anna*) [**142**] and steely-vented hummingbirds (*Saucerottia saucerottei*) [**143**] consume 0.20 and 0.19 g/kg/day of ethanol, respectively, given that nectar ethanol concentrations averaged 0.011% and sugar concentrations averaged 23.1% for all hummingbird-associated flower species. White-bellied sunbirds (*Cinnyris talatala*) [**143**], amethyst sunbirds (*Chalcomitra amethystina*) [**144**], and malachite sunbirds (*Nectarinia famosa*) [**145**] were estimated to consume 0.27, 0.20, and 0.19 g/kg/day of ethanol respectively, given that ethanol concentrations averaged 0.011% and sugar concentrations averaged 15.7% for the sunbird-associated flowers in this study. Lastly, honeybees (*Apis mellifera*) [**146**] were estimated to consume 0.05 g/kg/day of ethanol, given that the ethanol concentrations of insect-associated flowers averaged 0.011% and sugar concentrations averaged 30.5%.

3.4 Discussion

We found ethanol to be widespread within floral nectar, detected within at least one sample in 26 out of 29 species. Ethanol-positive samples were observed at a species average frequency of 51%, comparable to a 43% frequency of yeasts observed within flower nectar in a South African survey [**129**]. Ethanol concentrations, which averaged 0.011% (weight/weight) across species, were notably high in the family Cactaceae (0.024%, n=4 species; **Figure 3.1**) and had a weakly positive relationship with respect to sugar concentrations (**Figure 3.2**), supporting the hypothesis that ethanol could serve as a caloric signal to pollinators. The potential physiological significance of the estimated nectar ethanol values, which all fell below 0.06%, can be roughly illustrated for a given pollinator species using their published body mass and daily caloric intake estimates. As we show in **Table 3.1**, hummingbirds and sunbirds consume a daily dosage

Table 3.1: Estimated daily dosage of dietary ethanol of various animals. Ethanol dosage of birds and honeybees was calculated using daily energy intake and body mass from the referenced studies, with all energy assumed to be derived from nectar. The column for sample size (*n* samples) provides the numbers of individuals, males (M) and females (F) if that information was available. With the exception of hummingbird feeders, nectar sugar and ethanol concentrations for flowers visited by birds and honeybees are from the current study, with values corresponding to the respective pollinator class. Ethanol dosage values for pen-tailed treeshrews and chimpanzees are derived directly from the literature, with other data fields completed as available. To facilitate comparison, the dosage value of one standard alcoholic drink consumed by a 70 kg human is also provided. Note: although body mass is provided here in grams, to facilitate comparison the dosage is presented in grams per kilogram body mass.

Common name	Genus, species	substrate	*n* samples	Body mass (g)	Sugars (g)	Nectar sug. conc. (%)	Nectar consumed (g)	Ethanol (%w/w)	Ethanol (g)	Dosage (g/kg/day)	Reference (data)
Anna's hummingbird	*Calypte anna*	floral nectar	6M, 2F	4.48	1.98	23.1	8.6	0.011	0.0009	0.20	[**142**] (body mass and sugars)
Anna's hummingbird	*Calypte anna*	hummingbird feeder	6M, 2F	4.48	1.98	20.0	9.9	0.013	0.0013	0.30	[**137**] (ethanol and nectar sugar concentrations); [**142**] (body mass and sugars)
steely-vented hummingbird	*Saucerottia saucerottei*	floral nectar	6	4.13	1.75	23.1	7.6	0.011	0.0008	0.19	[**143**] (body mass and sugars)
white-bellied sunbird	*Cinnyris talatala*	floral nectar	6M, 2F	8.26	3.22	15.7	20.5	0.011	0.0022	0.27	[**144**] (body mass and sugars)
amethyst sunbird	*Chalcomitra amethystina*	floral nectar	4M, 5F	14.33	4.08	15.7	26.0	0.011	0.0028	0.20	[**144**] (body mass and sugars)
malachite sunbird	*Nectarinia famosa*	floral nectar	5	16.54	4.54	15.7	28.9	0.011	0.0031	0.19	[**145**] (body mass and sugars)
honeybee	*Apis mellifera*	floral nectar	13	0.101	0.012	30.5	0.040	0.011	0.000005	0.05	[**146**] (daily energy intake); [**159**] (body mass)

pen-tailed treeshrew	*Ptilocercus lowii*	floral nectar	12	47.00			38 mL	0.8	0.0658	1.40	[**41**] (dosage, body mass, % ethanol)
chimpanzee	*Pan troglodytes*	fruit		41,500					14.2	0.34	[**59**] (dosage, ethanol, body mass)
human	*Homo sapiens*	one standard alcoholic beverage		70,000					10	0.14	[**37**] (standard drink definition and rationale)

equivalent to 1.3–1.9 standard drinks in human terms [**59**], due in large part to their high estimated daily sugar requirements in relation to their body mass. Honeybees on the other hand were estimated to consume a quarter of the dosage of the larger avian pollinators, largely due to their lower estimated sugar requirements relative to body mass (**Table 3.1**). The observed nectar ethanol concentrations are also one order of magnitude lower than those previously recorded within the pulp of ripe fruits consumed by chimpanzees, which averaged 0.30% [**59**]. Fruit-eating chimpanzees consume only about 10–15% of their body mass per day in food, an order of magnitude less than sunbirds and hummingbirds, which leads to roughly the equivalent of 2.4 standard drinks in ethanol [**59**]. The ethanol concentrations reported here are comparable to those found previously within hummingbird feeders, which over a time period of weeks contained an average of 0.013% ethanol and contained comparable sugar concentrations of 20.0% [**137**]. In addition, these results are roughly two orders of magnitude lower than the 0.8% ethanol previously found in the nectaries of a tropical palm [**41**]. However, the daily dosage of ethanol per gram body mass over the course of a day of foraging is potentially substantial and of physiological relevance to these small-bodied pollinators given their large daily intake of nectar (**Table 3.1**).

The ethanol concentrations reported here can be compared to the concentrations of other common nectar compounds which have each been shown to attract pollinators at low doses and repel them at high ones, such as caffeine, nicotine and γ-aminobutyric acid (GABA). Ethanol-positive samples contained concentrations averaging 0.016% by species, equivalent to ~3.5 mM. Caffeine at concentrations of 0.1 mM improved the long-term memory of bees, which did not occur naturally within nectar above a concentration of 0.3 mM, and which served as a deterrent when above artificial concentrations of 1 mM [**7**]. Nicotine at naturally occurring concentrations under 0.02 mM were found to be attractive to bees [**160**], whereas natural floral nectar nicotine concentrations over 0.03 mM was found to be a deterrent to hummingbirds [**99**]. GABA, a commonly occurring amino acid and inhibitory neurotransmitter, was found to occur naturally at concentrations of 0.75 mM and was avoided by bumblebees at unnaturally high concentrations above 22 mM [**160**,**161**]. The ethanol concentrations we observed are therefore well within a range of concentrations of other compounds that can affect the foraging behaviors of pollinators. Here it is opportune to observe that all contemporary studies of the effect of ethanol on pollinator preference and physiology have examined much higher concentrations than those found here, with experimental treatment values starting as high as 0.31% (69 mM) [**162**], 1% [**137**,**163**,**164**], 1.25% [**138**], 2.5% [**165**–**167**], and 5% [**168**]. We recommend that future studies on the effect of dietary alcohol ingestion on pollinators incorporate low ethanol concentrations such as those presented here into their study design.

Here we, for the first time, successfully assayed volumetrically minute samples of floral nectar for ethanol concentrations at a very low detection threshold. We note four limitations to the precision and generalizability of these results and make recommendations for how future studies can potentially mitigate for them. First, the generalizability of these results is limited by our sampling opportunistically from a single botanical garden in a Mediterranean climate, which allowed us to compare different plant species exposed to a similar climate. Our observations are therefore unlikely to represent the full range of ethanol values found across a larger natural landscape under different ecological conditions, something future studies can address by sampling more broadly. Similarly, future studies should control for annual fluctuations across different seasons by sampling for longer periods of time. Second, although we took precautions to prevent evaporation of nectar samples during freezer storage, to control for evaporation during

prolonged storage future studies should freeze artificial nectar with known concentrations of sugar and ethanol alongside samples collected in the field. Third, although we excluded the results of many samples with noted color impurities (see **3.2 Materials and Methods**) there may be others with more subtle discoloration that we did not observe with the naked eye. Most such color impurities likely occurred due to accidentally scraping the flower nectary wall while probing with the microcapillary pipet, which is largely preventable with some dexterity, although the morphology of some flowers may make this difficult. Among the discarded results were six samples of *Fremontodendron californicum* which were all noted as 'cloudy white', and which all resulted in high ethanol concentrations of 0.110%, ten times the species average. Future studies could use a hemocytometer to investigate the microbial density of such samples to determine whether such discoloration could be attributed to relatively high microbial density rather than flower secretions. Future studies can also account for nectar discoloration by spectroscopically measuring all samples prior to assaying them, for example using a 96-well spectrophotometer. Fourth, due to low sampled nectar volumes we diluted all samples tenfold to achieve the minimum assay sample volume of 10 µL [**148**]. This in effect raised the detection threshold as specified in the assay instructions [**148**] tenfold to 0.008%, and all 60 samples with ethanol values below this cutoff were relabeled as zero. Future studies can avoid dilution by limiting sampling to flowers with higher nectar volumes or by pooling samples from multiple flowers. While there is no doubt this study shows ethanol is widespread within floral nectar, the aforementioned limitations make the precision of the reported estimates strictly preliminary. Future studies could validate the accuracy of this method by simultaneously assaying nectar ethanol using gas chromatography flame ionization detection (GC-FID) [**169**,**170**].

More work is needed to evaluate the physiological significance of these findings. Whereas the primary metabolic pathways of alcohol metabolism, namely the *alcohol dehydrogenase* (*ADH*) and *aldehyde dehydrogenase* (*ALDH*) family of genes and associated enzymes, are well-studied in humans and *Drosophila*, they are either unknown or poorly understood in other animals (but see [**171**–**174**]). Hummingbirds and honeybees tolerate much higher concentrations of ethanol than found here without exhibiting overt inebriation [**137**–**140,175**]. Thus, these pollinators are likely to have physiological mechanisms that allow them to efficiently metabolize the low but prevalent amounts of ethanol found in nectar.

Ethanol within floral nectar is assumed here to originate entirely from microbial fermentation. Plants also possess *ADH* genes and have been found in some cases to produce ethanol endogenously as a substrate for aroma biosynthesis [**176**], but no such mechanisms are known for nectar production. The low concentrations we observed are not entirely unexpected given the transient nature of most flowers along with the continual depletion and turnover of nectar and microbes that results from visitation by pollinators. However, this explanation alone fails to explain the similarly low concentrations found in feeders [**137**], which contain vastly higher volumes of (artificial) nectar and are presented for weeks on end. Alternatively, some limitations on fermentation and ethanol production might be imposed on yeasts by high osmotic stress associated with high sugar concentrations, resulting in energetic tradeoffs that lead to suppressed ethanol production, such as the biosynthesis of osmoprotectants [**177**].

Overall, our models explained ~30% of the variation in ethanol and 60% of the variation in sugar concentrations. The remaining unexplained variation in ethanol concentration might be affected by microbial diversity and abundance, or from factors such as temperature and pH. Sugar composition (i.e., the proportions of sucrose, glucose, and fructose) is known to be

influenced by microbial presence [**178**] and differs among pollinator syndromes [**179**], including between hummingbird- and sunbird-pollinated species [**180**]. Although not a significant variable in our models, ambient temperature has previously been shown to influence ethanol concentration within hummingbird feeders [**137**], and other environmental effects such as the amount of time the flower is illuminated, water availability, and soil conditions can also play a major role in nectar quality [**181**]. Future studies incorporating microbial assays and environmental effects at multiple sites could derive a more complete ecological understanding of the origins of ethanol within floral nectaries.

We did not exclude pollinators from any floral samples, and thus could not account for the amount of time a sample remained inoculated and unconsumed. Future work could experimentally exclude pollinators from some flowers (e.g., see [**182**]) so as to determine their influence on ethanol levels. Given that microbes can be vectored to nectaries by pollinators [**183**] pollinator consumption could potentially have strong effects on the timing of fermentation and the composition of microbes that colonize the flowers. Systematically tracking the ethanol concentrations within a virgin flower, after initial pollinator visitation, and then across its lifecycle to flower senescence could add much needed natural history context for this angiosperm-pollinator-yeast system. These data must be sampled carefully, however, to ensure that nectaries are not damaged during sample collection.

It is worth comparing the sugar concentrations we documented here to those found elsewhere to assess how the present data from a common garden relates to flowers in other natural landscapes. We documented sugar concentrations averaging 23.1% from floral species associated primarily with hummingbird pollination (n=12 species), which is a value well within the range of previous studies on hummingbird-pollinated flowers, which found average sugar concentrations of 21.0% in California (n=30) [**184**], and 25% (n=255) [**181**] and 24.3% (n=234) [**185**] more broadly across the Americas. In contrast, our average estimate of 15.7% sugar among six different sunbird-pollinated species was lower than documented elsewhere (21%; n=158) [**181**]. However, closer examination of three of the species individually shows that they are in fact close to expected sugar concentrations within their native range: *Greyia radlkoferi*, 9.2 vs 10.1% [**186**], *Melianthus major*, 12.7 vs. 12.2% [**187**], and *Kniphifia uvaria*, 12.3 vs 12.2% [**188**]. Thus, the difference in estimates of average sugar concentrations for sunbird-pollinated species is likely due to our much smaller sample size relative to [**181**]. Our value of 30.5% for insect-pollinated flowers is comparable to other published data (26.1%; n=20) [**185**], as well as for honeybee-pollinated flowers in California (31%; n=60) [**184**], but was lower than typical honeybee-pollinated flowers across the Americas as a whole (42.8%; n=145) [**189**]. Overall, the sugar concentrations that we measured were comparable to sugar concentrations measured across many different environments.

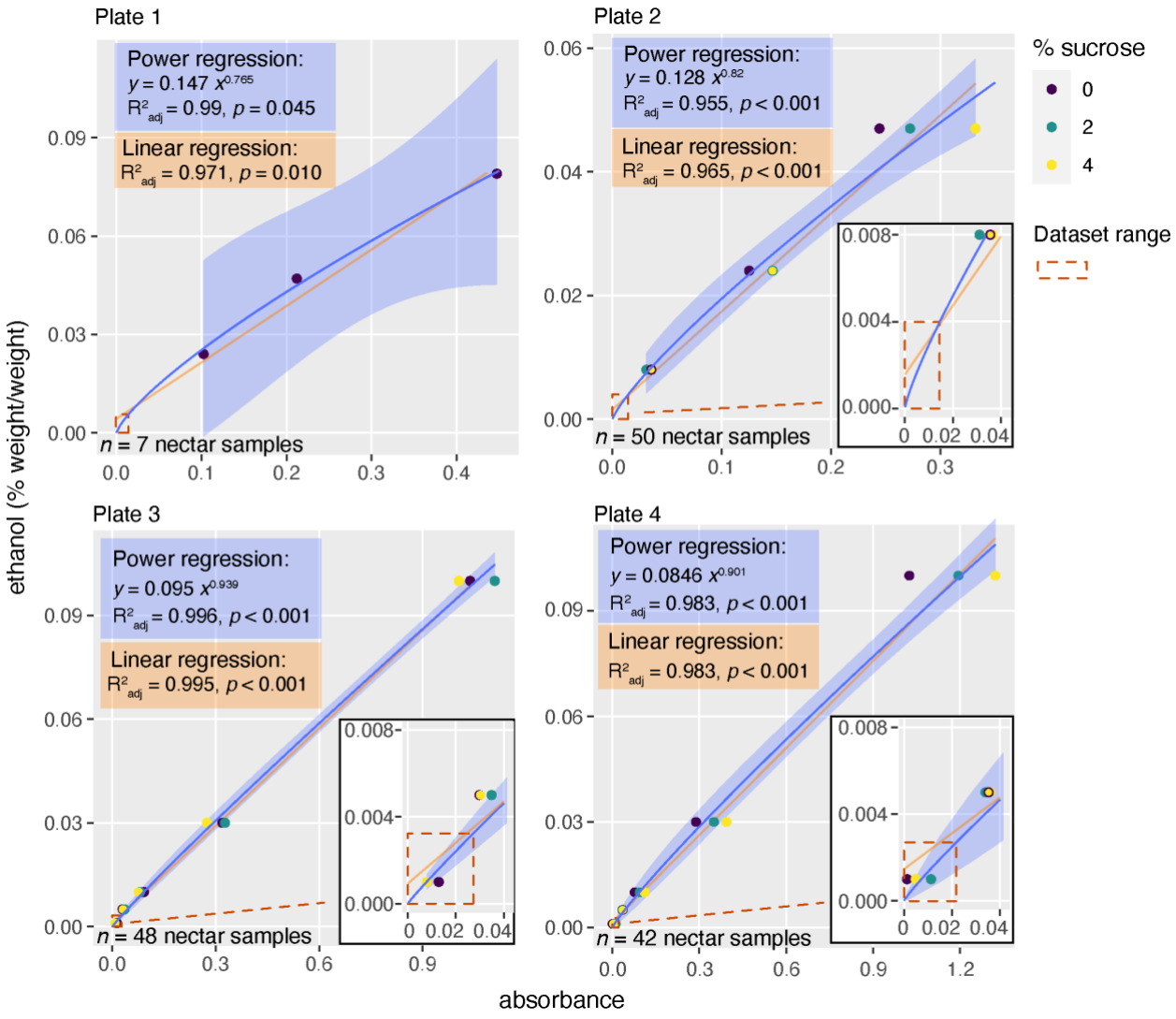
Given our observations of widespread ethanol occurrence, more work is needed to determine if ethanol within flowers plays an olfactory role in pollinator ecology, as is the case in several other systems. For example, fruit flies (*Drosophila melanogaster*) have previously been shown to be able to track ethanol plumes to their source [**190**]. Likewise, a variety of wood boring beetles are known to be attracted to ethanol produced by anaerobic respiration within the bark of diseased or dying hardwood trees [**191**,**192**]. Although electroantennographic assays of honeybee antennae exposed to the volatile emissions of nectar-isolated microbes resulted in a relatively small olfactory response to ethanol, bee antennae were shown to be sensitive to several other products of ethanolic fermentation [**122**]. Detection of nectar microbe volatile emissions

via olfaction can potentially enable pollinators to differentiate among flowers inoculated with yeasts and bacteria, possibly saving time in the assessment of nectar quality without direct floral contact.

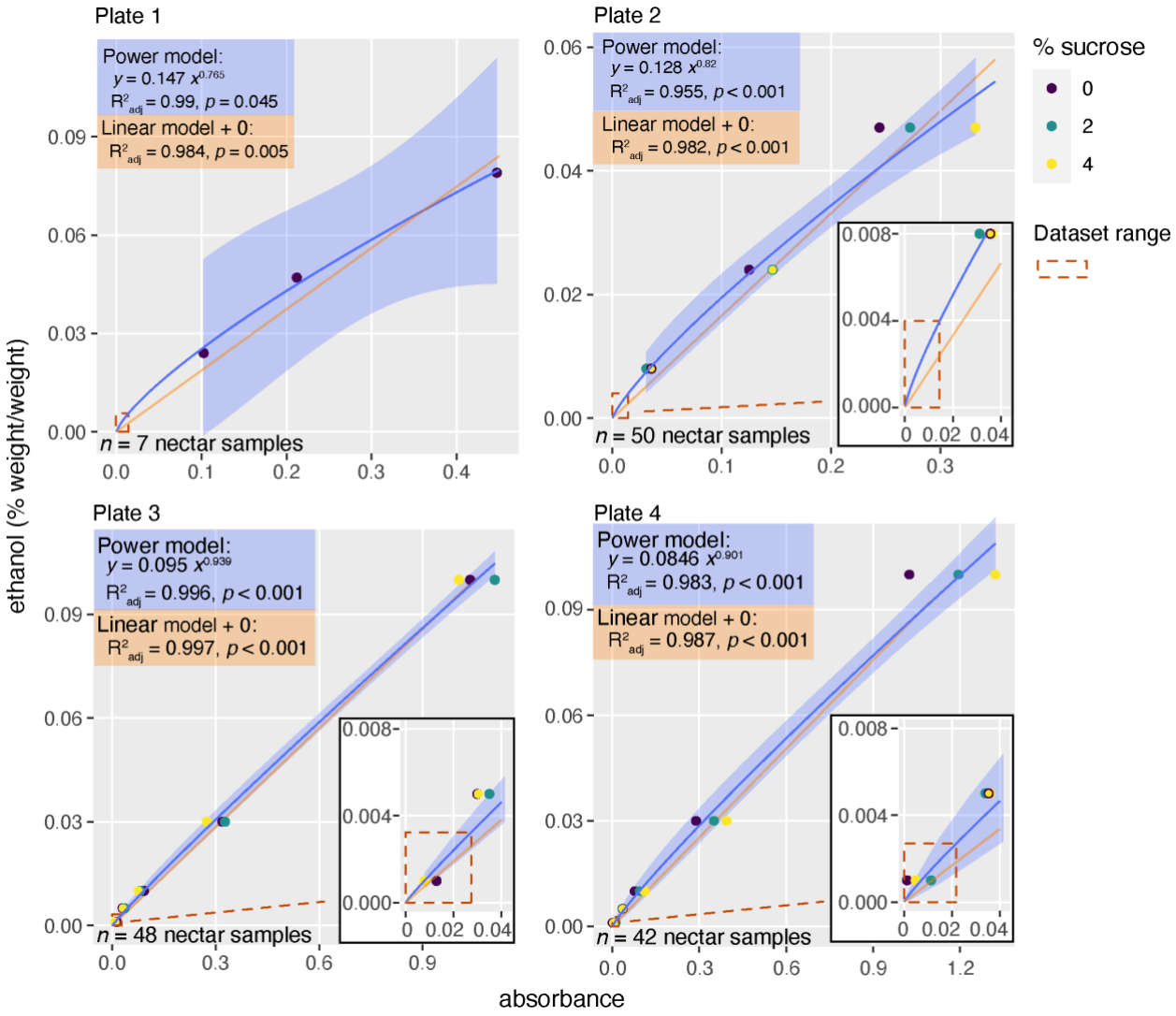
Nectar ethanol may have further implications for the ecology of nectarivores via their microbiome. For example, the fruit fly's gut microbiome attenuates some of the negative effects of ethanol exposure on lifespan and fecundity [**105**]. Variations in the honeybee crop microbiome may result in the endogenous accumulation of ethanol [**193**]. Of particular interest are the acetic acid bacteria (Acetobacteraceae) endosymbionts, which associate with the insect midgut but also colonize host tissues and organs, and which are common among several orders of insects with a sugar-based diet, including honeybees [**194**]. Acetic acid bacteria convert wine into vinegar, and occupy an ecological niche that leverages consumption of ethanol over sugars [**194**]. Interestingly, these bacteria (namely *Asaia* sp. and *Neokomagatea* sp.), are routinely found within floral nectar [**129**,**195**,**196**] and hummingbird feeders [**197**], with both genera found almost exclusively in association with ethanol-producing yeasts within floral nectar [**122**,**129**].

We have for the first time documented ethanol concentrations within nectar for a wide diversity of flowers grown in common garden conditions. We found that ethanol is widespread in floral nectars at low concentrations, but at levels that may be physiologically relevant for those pollinators that consume large amounts of nectar relative to their body mass. Further studies of dietary preference by nectarivores should consider testing species with the naturally occurring but low ethanol concentrations observed within nectar. Finally, the ecological role of nectar fermentation is understudied given its apparent ubiquity. Future studies of ethanol levels in nectar should evaluate broader features of flower morphology, pollinator composition, and microbial diversity and abundance on levels of ethanol in addition to sugar composition and concentration.

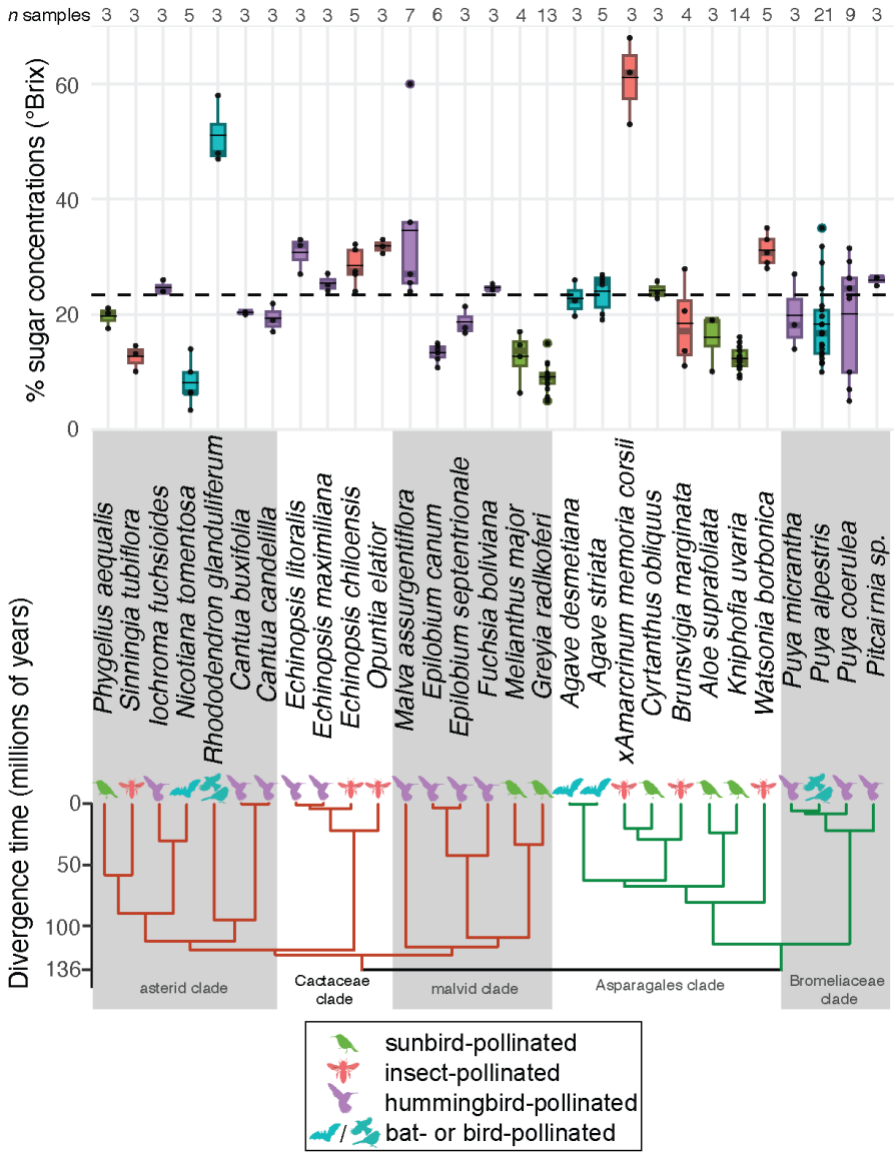
3.5 Appendix



Supplementary figure S3.1. Calibration regressions of ethanol standards for four separate assays (plates 1–4), comparing a power regression (blue) and **a linear regression (orange)**. The dashed red square represents the actual data range of assayed nectar samples using the power regression to facilitate visual comparison of the regression fit relative to low concentration calibration standards. Points are colored to correspond to the concentration of sugars added to some standards.



Supplementary figure S3.2. Calibration regressions of ethanol standards for four separate assays (plates 1–4), comparing a power regression (blue) and **a linear regression forced through the intercept (orange).** The dashed red square represents the actual data range of assayed nectar samples using the power regression to facilitate visual comparison of the regression fit relative to low concentration calibration standards. Points are colored to correspond to the concentration of sugars added to some standards.



Supplementary figure S3.3. Sugar concentrations in floral nectar. Average sugar concentrations of floral nectar for 29 angiosperm species arranged according to phylogenetic relatedness [**32**]. Green branches on the phylogeny refer to monocot species, and orange branches refer to eudicot species. Groups of species are classified into clades as indicated by gray alternating with white shading, as labeled at the bottom of the chart. Boxplots are color-coded by the expected pollinator for that plant species, with corresponding animal silhouettes (from PhyloPic) at the phylogeny tips: purple = hummingbird-pollinated (trochilophily), green = sunbird-pollinated (nectariniophily), salmon = insect-pollinated (entomophily), and cyan = "other" (including chiropterophily, ornithophily, and general zoophily). A black dashed line demarcates the average sugar concentration of 23.4% among all species. Boxplots represent the 25th to 75th percentiles of the interquartile range (IRQ), a thick line color-coded line represents the median, and whiskers represent the minimum and maximum. The median, boxplot and whisker calculations exclude outliers, which are shown as points and represent ethanol values greater than the IRQ multiplied by 1.5, as added to the 75th percentile. Sample size (*n* samples) is indicated above each boxplot.

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