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9-Borobicyclo[3.3.1]nonane-Catalyzed Hydroboration of Terminal Aromatic Alkynes with Pinacolborane

*Garett L. Ruesch, Sydney L. Rowley, Marcus C. Mifflin, & Nathan S. Werner**
Department of Physical Science, Southern Utah University, Cedar City, UT

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Students: garett.ruesch@gmail.com, sydney.rowley.17@gmail.com, mifflinma@gmail.com
*Mentor: nathanwerner@suu.edu**

ABSTRACT
Organoboron compounds are extensively used in organic synthesis. The alkenylboronic acid pinacol esters formed from the hydroboration reaction of alkynes with pinacolborane are stable, easy to handle, and useful in many synthetic transformations. However, pinacolborane lacks the reactivity necessary to undergo facile hydroboration reaction with terminal aromatic alkynes. 9-Borobicyclo[3.3.1]nonane (9-BBN) can be used to catalyze the hydroboration reaction of phenylacetylene with pinacolborane. The hydroboration reaction parameters and product purification conditions were evaluated to maximize the yield of (*E*)-2-phenylethenylboronic acid pinacol ester. It was found that the optimal reaction conditions for the 9-BBN-catalyzed hydroboration of phenylacetylene with pinacolborane were: phenylacetylene (1.0 equiv), pinacolborane (1.2 equiv), 9-BBN (20 mol%), and THF [0.2] at 65 °C. The compatibility of these reaction conditions with *p*-substituted terminal aromatic alkynes bearing electronically diverse groups was studied. Moderate to good yield (49–76%) of the hydroboration products were isolated after purification by liquid-liquid extraction and flash chromatography.

KEYWORDS
Organic Synthesis; Catalysis; Methods Development; Hydroboration; Reaction Optimization; Alkenylboronic Ester; Alkyne; Pinacolborane; 9-Borobicyclo[3.3.1]nonane

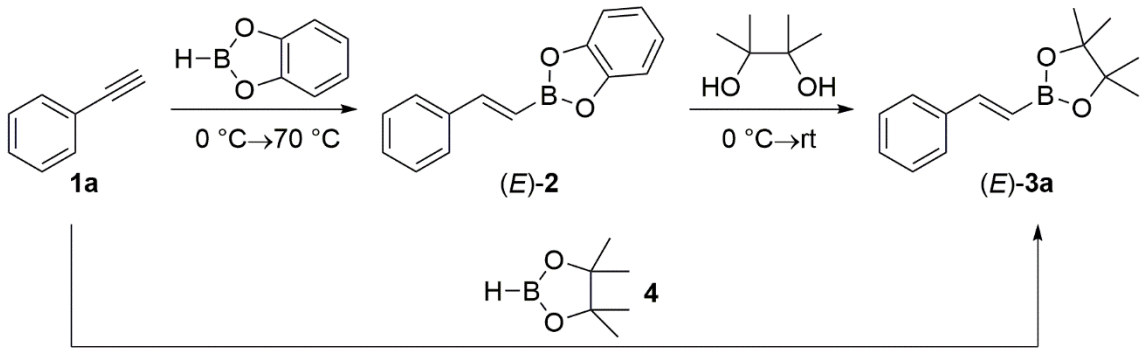
INTRODUCTION

Boron containing organic compounds are useful reagents in the synthesis of important carbon-containing medicines, materials, and fine chemicals. The utility of these reagents is evident from the variety of bonds, and therefore functional groups, which can be substituted for the C–B bond. For example, the hydroboration-oxidation reaction of an alkene ultimately transforms the C–B bond into a C–O bond.[1] The C–B bond can be substituted for a C–H bond by protonolysis,[2] or a C–X bond by halogenation.[3] In addition, the use of transition-metal catalysis has allowed for the substitution of a C–B bond for a C–C,[4, 5, 6, 7] C–H,[8, 9] C–N,[10, 11, 12, 13, 14] C–O,[15, 16, 17] C–P,[18, 19] or C–S bond,[20, 21] and include asymmetric variants.[22]

The hydroboration reaction of unsaturated organic substrates with hydroboron reagents is a straight-forward method to prepare alkyl- and alkenylboron compounds. An early example of the hydroboration reaction of alkenes was reported by Brown in 1956.[1, 23] In 1966, Woods and Strong reported that alkynes undergo sluggish hydroboration with 4,4,6-trimethyl-1,3,2-dioxaborinane in a sealed tube of superheated ether to provide the alkenylboron products in low yield.[24] Chemists have sought to catalyze the hydroboration reaction with transition metals,[25, 26, 27, 28, 29, 30, 31, 32] and alkaline earth metals,[33, 34] aluminum,[35, 35, 36, 37] base,[38, 39] *N*,*N*-dimethylacetamide,[40] and benzoic acid derivatives.[41, 42] Boron reagents are also known to catalyze the hydroboration reaction.[43, 44, 45, 46, 47, 48] In 1990, Periasamy *et al.* reported that a BH_3·*N*,*N*-diethylaniline complex could catalyze the hydroboration of terminal alkynes with catecholborane.[43] Hoshi *et al.* later reported that dicyclohexylborane could catalyze the hydroboration reaction of terminal alkynes with pinacolborane in good to excellent yield.[44]

Our interest in the hydroboration reaction originated with the need to synthesize (*E*)-2-phenylethenyl boronic acid pinacol ester (*E*)-**3a** (Scheme 1) for use as an organometallic donor in Suzuki-Miyaura cross-coupling reactions.[7] The reagent (*E*)-**3a** could be synthesized by hydroboration reaction with catecholborane followed by diol exchange with pinacol.[51] However, the use of catecholborane is not ideal. For example, catechol is sensitive to oxidation, and the catecholborane product can decompose upon exposure to air or moisture.[52] To avoid the use of catechol, a direct hydroboration reaction of **1a** with pinacolborane **4** was sought. The reagent **4** is commercially available and more stable than catecholborane. Although the increased stability of **4** also lessens its reactivity in the hydroboration reaction. For example, the hydroboration reaction of **1a** (1 equiv) with **4** (1.2 equiv) is sluggish in refluxing THF (65 °C), and only trace amounts of product (*E*)-**3a** was observed after 7 h *vide infra*. It was found that 9-borobicyclo[3.3.1]nonane (9-BBN) could be used to catalyze the hydroboration reaction of **1a** with **4**.[7] The initially evaluated

reaction conditions were determined to be satisfactory at the time and were not optimized. The reaction parameters and purification conditions have since been studied in detail and are described here.



Scheme 1.

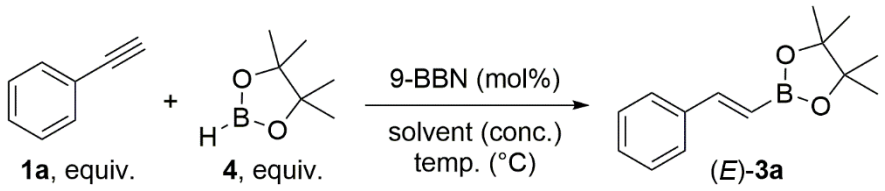
RESULTS AND DISCUSSION

This study began with the use of the previously published reaction conditions: **1a** (1.0 equiv), **4** (1.0 equiv), 9-BBN (10 mol%) in refluxing 0.2 M THF solution (Table 1).[7] On a 1 mmol scale, the hydroboration product (*E*)-**3a** was isolated from this reaction as a slightly yellow oil in 62% yield after purification by liquid-liquid extraction and flash chromatography (entry 1). The effect of reagent stoichiometry on the yield of (*E*)-**3a** was then evaluated. Increasing the amount of **4** to 1.2 equiv increased the yield to 73% (entry 2). Further increasing the amount of **4** to 1.4 equivalents decreased the yield of (*E*)-**3a** (entry 3). The use of excess **1a** (1.2 equiv) and **4** (1.0 equiv) as the limiting reactant provided a 69% yield of (*E*)-**3a** (entry 4). A range from 0-30 mol% of 9-BBN was tested, and 20 mol% was found to provide the highest yield of (*E*)-**3a** (entries 12-15).

The reaction parameters of concentration, temperature, and a NaOH additive were also evaluated. It was found that concentrations between 0.2 M and neat[49] caused little change in the amount of (*E*)-**3a** that was isolated (entries 5–7). However, a more dilute reaction (0.04 M) produced significantly less (*E*)-**3a** (entry 5). Toluene and 1,4-dioxane were evaluated as solvent and were found to provide a lower yield than when THF was used as the reaction solvent (entries 10 and 11). A decreased reaction temperature resulted in a decreased yield of (*E*)-**3a** (entry 8). An increased reaction temperature resulted in an increased yield of (*E*)-**3a** when 1,4-dioxane was used as solvent (entries 9 and 10). However, the yield of (*E*)-**3** was higher when the reaction was run in THF at 65 °C than when the reaction was run in 1,4-dioxane at 85 °C (compare entries 2 and 10). The addition of NaOH (5 mol%) resulted in a slightly decreased yield of (*E*)-**3a** (entry 16).[38]

Various stationary phases were evaluated for the purification of (*E*)-**3a** by flash chromatography. The use of a boric acid capped silica provided a negligible difference in yield and purity as determined by ^{1}H NMR (entry 17).[50] The crude product (*E*)-**3a** was entirely lost when the flash chromatography was conducted with a dry, neutral alumina stationary phase (entry 18).

Table 1. Evaluation of the reaction parameters of the 9-BBN-catalyzed hydroboration reaction of **1a** with **4**.

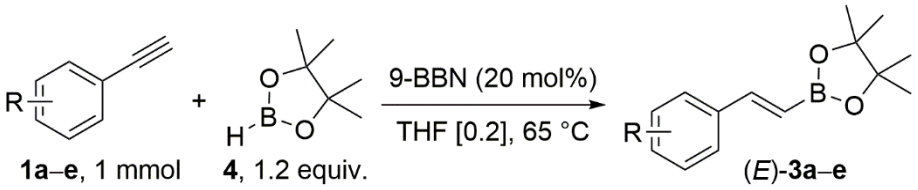


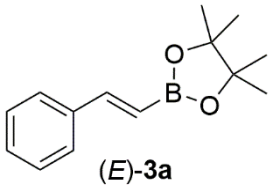
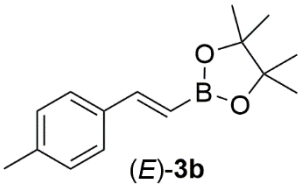
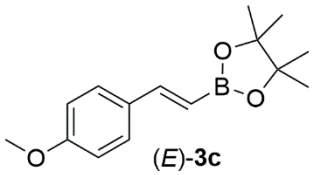
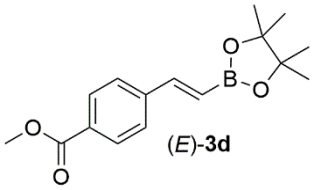
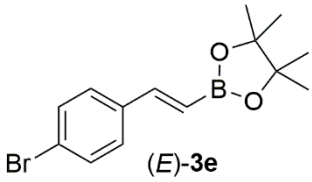
entry	HB(pin), mmol	9-BBN, mol%	solvent, conc.	temp., °C	yield,[a] %
1	1	10	THF, 0.2	65	62
2	1.2	10	THF, 0.2	65	73
3	1.4	10	THF, 0.2	65	67
4[b]	1	10	THF, 0.2	65	69
5	1.2	10	THF, 0.04	65	53
6	1.2	10	THF, 1.0	65	71
7	1.2	10	neat[c]	65	69
8	1.2	10	THF, 0.2	45	54
9	1.2	10	dioxane, 0.2	65	48
10	1.2	10	dioxane, 0.2	85	59
11	1.2	10	toluene, 0.2	65	54
12	1.2	–	THF, 0.2	65	0[d]
13	1.2	5	THF, 0.2	65	46
14	1.2	20	THF, 0.2	65	76
15	1.2	30	THF, 0.2	65	71
16[e]	1.2	10	THF, 0.2	65	66
17[f]	1.2	10	THF, 0.2	65	63
18[g]	1.2	10	THF, 0.2	65	0

[a]Yield of isolated, purified product.
[b]1.2 equiv of phenylacetylene was used.
[c]9-BBN was used as a 0.5 M solution in THF. The final reaction concentration was 2.5 M.
[d]No product was observed after 7 h.
[e]NaOH (5 mol%) was added as a co-catalyst.
[f]Borated silica was used as the chromatography stationary phase.
[g]Dry, neutral alumina was used as the chromatography stationary phase

The optimized reaction conditions of **1** (1.0 equiv), **4** (1.2 equiv), 9-BBN (20 mol%), and THF [0.2] at 65 °C were then used to evaluate the scope of compatible *p*-substituted terminal aromatic alkynes **1a–e**. The electronically neutral parent alkyne **1a** provided the highest yield (76%) and was found to react the fastest under the optimized reaction conditions (entry 1). Substrates with electron-donating methyl and methoxy groups provided a good yield of the desired products (*E*)-**3b** and (*E*)-**3c** (entries 2 and 3). A substrate bearing an electron-withdrawing methyl ester group provided the lowest yield of the substrates evaluated (entry 4). The good yield of the bifunctional, 4-bromo derivative (*E*)-**3e** is especially interesting because it could be used as an organic electrophile or organometallic donor in a Suzuki-Miyaura cross-coupling reaction. Complete control of the alkene geometry of (*E*)-**3a–e** was observed by ^{1}H NMR spectroscopy in all of the cases studied.

Table 2. Evaluation of the optimized reaction conditions with **1a** and electronically diverse *p*-substituted terminal aromatic alkynes **1b**–**1e**.



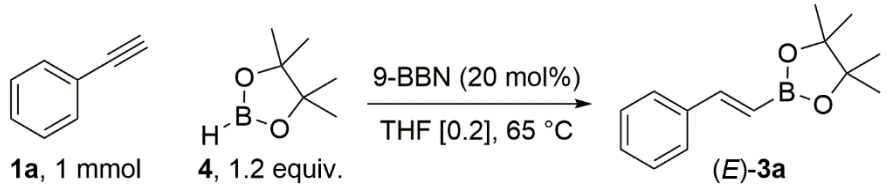
entry	product	yield %	reaction time (h)
1	 (*E*)-**3a**	76%	1.5
2	 (*E*)-**3b**	64%	2
3	 (*E*)-**3c**	63%	3
4	 (*E*)-**3d**	49%	7
5	 (*E*)-**3e**	72%	3

METHODS AND PROCEDURES

General Procedure: An oven-dried, round-bottom flask equipped with a magnetic stir-bar was fitted with an oven-dried Liebig condenser and sealed with high-vacuum grease. The reflux apparatus was capped with a septum and purged with a balloon of argon that was introduced and evacuated through a needle for 15 minutes. The following were added sequentially by syringe under a balloon of argon: the terminal aromatic alkyne, pinacolborane, 9-BBN ([0.5] in THF), and finally a small portion of solvent to wash all of the reagents into the round-bottomed flask. The reaction solution was refluxed in a preheated oil bath at 65 °C. Reaction aliquots were analyzed by thin-layer chromatography (TLC) to monitor the disappearance of the alkyne starting material. When the reaction was determined to be complete, it was cooled to room temperature, extracted with ethyl acetate, washed with water, brine, dried with sodium sulfate, filtered and concentrated by rotary evaporation (50 °C, 2 torr). The crude product was purified by flash chromatography on silica. The amount of silica used in the chromatography was approximately 65 times the mass of the concentrated crude product. The eluent began with 98.5:1.5, hexane/ethyl acetate and increased to 95:5,

hexane/ethyl acetate until the product was completely eluted. The product was concentrated *in vacuo* (2 torr) to afford the desired product.

*Procedure for the Synthesis of 4,4,5,5-Tetramethyl-2-[(1E)-2-phenylethenyl]-1,3,2-dioxaborolane ((E)-**3a**)*



Following the General Procedure, phenylacetylene (110 μL, 1.0 mmol, 1.0 equiv), pinacolborane (174 μL, 1.2 mmol, 1.2 equiv), 9-BBN (0.400 mL, [0.5], 0.2 mmol, 0.2 equiv), and THF (5 mL) were combined and heated to 65 °C for 1.5 h. Purification by aqueous workup and flash chromatography afforded 175 mg (76%) of (*E*)-**3a** as a slightly yellow oil.[38]

Data *for 4,4,5,5-Tetramethyl-2-[(1E)-2-phenylethenyl]-1,3,2-dioxaborolane ((E)-**3a**)*:

^{1}H NMR: (400 MHz, $CDCl_3$)
7.48–7.45 (m, 2 H), 7.40 (d, *J* = 18.5, 1 H), 7.33–7.23 (m, 3 H) 6.17 (d, *J* = 18.5, 1 H), 1.29 (s, 12 H).

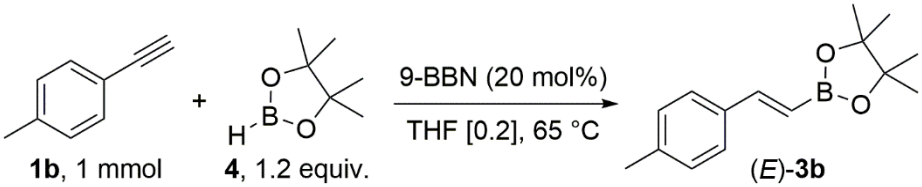
^{13}C NMR: (101 MHz, $CDCl_3$)
149.5, 137.5, 128.9, 128.6, 127.1, 83.4, 24.9.

IR: (neat)
3080 (w), 3054 (w), 3018 (w), 2978 (w), 2928 (w), 1623 (m), 1577 (w), 1495 (w), 1450 (m), 1391 (m), 1371 (m), 1346 (s), 1322 (s), 1271 (m), 1237 (m), 1210 (m), 1142 (s), 1109 (w), 997 (m), 969 (m), 899 (m), 851 (m), 748 (m), 692 (m), 660 (w), 641 (w).

MS: (EI, 70 eV)
230 ($[M]^+$, 49), 229 (12), 215 (24), 157 (11), 145 (38), 144 (69), 143 (10), 131 (56), 130 (100), 129 (92), 118 (16), 114 (14), 105 (31), 104 (15), 103 (16), 85 (10), 78 (12), 77 (21).

TLC: R_f 0.53 (hexane/ethyl acetate, 95:5) [silica gel, aqueous $KMnO_4$]

*Procedure for the Synthesis of 4,4,5,5-Tetramethyl-2-[(1E)-2-(4-methylphenyl)ethenyl]-1,3,2-dioxaborolane ((E)-**3b**)*



Following the General Procedure, 4-tolylacetylene (127 μL, 1.0 mmol, 1.0 equiv), pinacolborane (174 μL, 1.2 mmol, 1.2 equiv), 9-BBN (0.400 mL, [0.5], 0.2 mmol, 0.2 equiv), and THF (5 mL) were combined and heated to 65 °C for 2 h. Purification by aqueous workup and flash chromatography afforded 157 mg (64%) of (*E*)-**3b** as a slightly yellow oil.[37]

*Data for 4,4,5,5-Tetramethyl-2-[(1E)-2-(4-methylphenyl)ethenyl]-1,3,2-dioxaborolane ((E)-**3b**)*

^{1}H NMR: (400 MHz, $CDCl_3$)
7.40–7.38 (m, 2 H), 7.38 (d, *J* = 18.5, 1 H), 7.15–7.13 (m, 2 H), 6.11 (d, *J* = 18.5, 1 H), 2.34 (s, 3 H), 1.31 (s, 12 H).

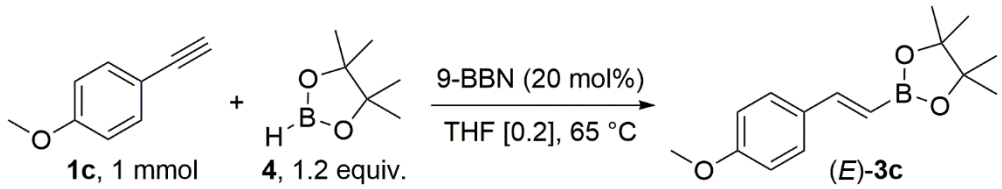
^{13}C NMR: (101 MHz, $CDCl_3$)
149.6, 139.1, 134.9, 129.4, 127.2, 83.4, 25.0, 21.5.

IR: (neat)
2976 (w), 2926 (w), 1625 (m), 1569 (w), 1511 (w), 1479 (w), 1451 (w), 1411 (w), 1379 (m), 1370 (m), 1345 (m), 1320 (s), 1285 (w), 1264 (w), 1220 (w), 1204 (w), 1178 (w), 1165 (w), 1138 (s), 1109 (m), 1019 (s), 999 (m), 969 (m), 950 (w), 901 (w), 853 (w), 843 (m), 829 (w), 796 (s), 759 (w), 712 (w), 676 (w), 659 (w), 642 (w), 580 (w), 517 (w), 492 (m), 453 (w).

MS: (EI, 70 eV)
245 ($[M+1]^+$, 12), 244 ($[M]^+$, 69), 243 ($[M-1]^+$, 17), 229 (21), 171 (12), 159 (40), 158 (54), 145 (38), 144 (92), 143 (100), 132 (19), 128 (47), 127 (13), 119 (17), 118 (12), 117 (43), 116 (29), 115 (36), 91 (18), 43 (15), 41 (17), 40 (11).

TLC: R_f 0.42 (hexane/ethyl acetate, 95:5) [silica gel, aqueous $KMnO_4$]

*Procedure for synthesis of 2-[(1E)-2-(4-Methoxyphenyl)ethenyl]-4,4,5,5-tetramethyl-1,3,2-dioxaborolane ((E)-**3c**)*



Following the General Procedure, 4-ethynylanisole (130 mg, 1.0 mmol, 1.0 equiv), pinacolborane (174 μL, 1.2 mmol, 1.2 equiv), 9-BBN (0.400 mL, [0.5], 0.2 mmol, 0.2 equiv), and THF (5 mL) were combined and heated to 65 °C for 3 h. Purification by aqueous workup and flash chromatography afforded 132 mg (51%) of (*E*)-**3c** as a slightly yellow oil.[46]

*Data for 2-[(1E)-2-(4-Methoxyphenyl)ethenyl]-4,4,5,5-tetramethyl-1,3,2-dioxaborolane ((E)-**3c**)*

^{1}H NMR: (400 MHz, $CDCl_3$)
7.45–7.42 (m, 2 H), 7.35 (d, *J* = 18.4, 1 H), 6.88–6.85 (m, 2 H), 6.01 (d, *J* = 18.4, 1 H), 3.81 (s, 3 H), 1.31 (s, 12 H).

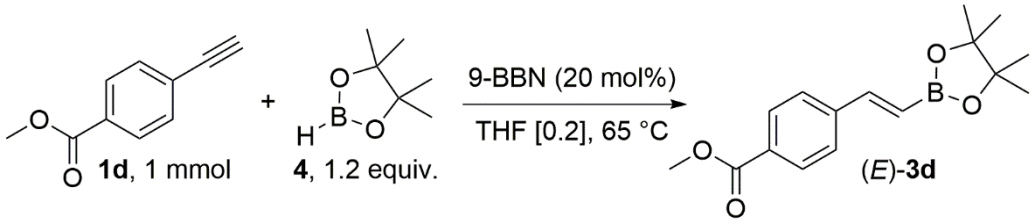
^{13}C NMR: (101 MHz, $CDCl_3$)
160.4, 149.2, 130.6, 128.6, 114.1, 83.4, 55.4, 25.0.

IR: (neat)
2976 (w), 2932 (w), 2836 (w),1697 (w), 1624 (w), 1624 (w), 1603 (m), 1575 (w), 1509 (m), 1457 (w), 1379 (w), 1370 (w), 1351 (m), 1319 (m), 1303 (m), 1291 (w), 1250 (m), 1210 (m), 1194 (w), 1170 (m),1140 (m), 1105 (w), 1032 (m), 995 (w), 969 (m), 900 (w), 852 (m), 814 (m), 758 (w), 734 (w), 720 (w), 699 (w), 675 (w), 644 (w), 607 (w), 607 (w), 597 (w), 578 (w), 541 (w), 519 (m), 450 (w).

MS: (EI, 70 eV)
261 ([M+1]$^+$, 17), 260 ([M]$^+$, 100), 259 ([M-1]$^+$, 24), 245 (17), 175 (30), 174 (19), 161 (45), 160 (78), 159 (47), 148 (15), 146 (10), 145 (23), 144 (99), 143 (28), 135 (15), 121 (16), 117 (24), 77 (12), 43 (13), 41 (15), 40 (14).

TLC: R_f 0.26 (hexane/ethyl acetate, 95:5) [silica gel]

*Procedure for the Synthesis of 4-[(1E)-2-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)ethenyl]benzoic acid methyl ester ((E)-**3d**)*



Following the General Procedure, methyl 4-ethynylbenzoate (160 mg, 1.0 mmol, 1.0 equiv), pinacolborane (174 μL, 1.2 mmol, 1.2 equiv), 9-BBN (0.400 mL, [0.5], 0.2 mmol, 0.2 equiv), and THF (5 mL) were combined and heated to 65 °C for 7 h. Purification by aqueous workup and flash chromatography afforded 149 mg (52%) of (*E*)-**3d** as off-white crystals.[46]

*Data for 4-[(1E)-2-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)ethenyl]benzoic acid methyl ester ((E)-**3d**)*

^{1}H NMR: (400 MHz, $CDCl_3$)
8.00–7.98 (m, 2 H), 7.53–7.51 (m, 2 H), 7.40 (d, *J* = 18.4, 1 H), 3.89 (s, 3 H), 1.30 (s, 12 H).

^{13}C NMR: (101 MHz, $CDCl_3$)
166.8, 148.2, 141.8, 130.2, 130.0, 127.0, 83.6, 52.2, 24.9.

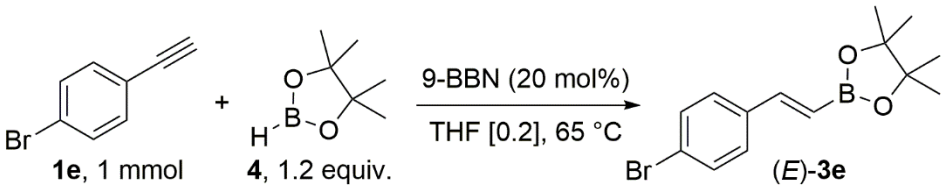
IR: (neat)
2975 (w), 2950 (w), 2931 (w), 2848 (w), 1712 (s), 1672 (w), 1627 (m), 1605 (m), 1566 (w), 1487 (w), 1441 (m), 1413 (w), 1380 (w), 1368 (m), 1349 (s), 1319 (s), 1275 (s), 1214 (m), 1195 (m), 1173 (m), 1149 (m), 1111 (s), 1015 (w), 1002 (m), 972 (m), 902 (w), 872 (m), 855 (m), 844 (m), 835 (m), 804 (w), 758 (s), 703 (m), 690 (m), 667 (w), 652 (w), 638 (m), 630 (m), 579 (w), 518 (w), 508 (m), 487 (w), 479 (w), 451 (w).

MS: (EI, 70 eV)
288 ([M]$^+$, 49), 287 ([M-1]$^+$, 15), 273 (32), 257 (19), 203 (29), 202 (57), 189 (20), 188 (30), 187 (53), 176 (18), 172 (13), 171 (15), 158 (11), 157 (100), 156 (32), 143 (45), 130 (11), 129 (39), 128 (17), 77 (29), 59 (15), 43 (20), 41 (21).

MP: 108-109 °C

TLC: R_f 0.18 (hexane/ethyl acetate, 95:5) [silica gel, aqueous $KMnO_4$]

*Procedure for the Synthesis of 2-[(1E)-2-(4-Bromophenyl)ethenyl]-4,4,5,5-tetramethyl-1,3,2-dioxaborolane ((E)-**3e**)*



Following the General Procedure, 1-bromo-4-ethynylbenzene (309 mg, 1.0 mmol, 1.0 equiv), pinacolborane (174 μL, 1.2 mmol, 1.2 equiv), 9-BBN (0.400 mL, [0.5], 0.2 mmol, 0.2 equiv), and THF (5 mL) were combined and heated to 65 °C for 3 h. Purification by aqueous workup and flash chromatography afforded 220 mg (71%) of (*E*)-**3e** as white crystals.[37]

*Data for 2-[(1E)-2-(4-Bromophenyl)ethenyl]-4,4,5,5-tetramethyl-1,3,2-dioxaborolane ((E)-**3e**)*

^{1}H NMR: (400 MHz, $CDCl_3$)
7.47–7.44 (m, 2 H), 7.36–7.32 (m, 2 H), 7.32 (d, *J* = 18.5, 1 H), 6.14 (d, *J* = 18.5, 1 H), 1.31(s, 12 H).

^{13}C NMR: (101 MHz, $CDCl_3$)
148.2, 136.6, 131.9, 128.7, 123.0, 83.6, 25.0.

IR: (neat)
3087 (w), 3059 (w), 3046 (w), 3007 (w), 2973 (w), 2929 (w), 2290 (w), 1906 (w), 1701 (w), 1624 (m), 1583 (w), 1564 (w), 1486 (m), 1467 (w), 1401 (m), 1378 (w), 1370 (w), 1343 (m), 1319 (s), 1301 (m), 1296 (m), 1268 (m), 1209 (m), 1165 (w), 1138 (m), 1110 (m), 1067 (m), 1006 (m), 991 (m), 968 (m), 952 (w), 899 (w), 852 (m), 820 (w), 799 (s), 706 (w), 693 (w), 667 (w), 653 (w), 638 (m), 628 (w), 598 (w), 579 (w), 515 (s), 485 (w), 453 (w).

MS: (EI, 70 eV)
310 ([M+2]$^+$, 50), 309 ([M+1]$^+$, 21), 308 ([M]$^+$, 51), 307 ([M-1]$^+$, 14), 295 (22), 293 (22), 225 (22), 224 (48), 223 (24), 222 (47), 211 (27), 210 (85), 209 (65), 208 (89), 207 (39), 198 (16), 196 (17), 194 (23), 192 (23), 144 (31), 143 (100), 139 (10), 131 (10), 130 (59), 129 (92), 128 (47), 103 (14), 102 (23), 101 (11), 85 (21), 77 (55), 76 (12), 75 (11), 59 (13), 57 (12), 43 (34), 42 (11), 41 (36), 40 (39).

MP: 78-79 °C

TLC: R_f 0.42 (hexanes/ethyl acetate, 95:5) [silica gel, aqueous $KMnO_4$]

CONCLUSION

(*E*)-2-phenylethenyl boronic acid pinacol ester derivatives can be readily prepared by 9-BBN-catalyzed hydroboration reaction of terminal aromatic alkynes with pinacolborane. The use of 20 mol% of 9-BBN was found to provide the best yield of the hydroboration product. A variety of electronically diverse substrates provided moderate to good yields of the (*E*)-2-phenylethenyl boronic acid pinacol ester derivatives (*E*)-**3a–e**. The bifunctional, 4-bromo derivative is especially interesting because it could be used as an organic electrophile or organometallic donor in a cross-coupling reaction. Complete control of the alkene geometry of the alkenylboronic ester product was observed in all of the cases studied. We intend to continue to explore the scope of this reaction and the synthetic utility of the boronic acid pinacol ester products.

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REFERENCES

1. Subba Rao, B. C., Brown, H. C. (1956) A new technique for the conversion of olefins into organoboranes and related alcohols, *J Am Chem Soc* 78, 5694–5695. *https://doi.org/10.1021/ja01602a063*
2. Brown, H. C., Murray, K. (1959) Convenient non-catalytic conversion of olefinic derivatives into saturated compounds through hydroboration and protonolysis, *J Am Chem Soc* 81, 4108–4109. *https://doi.org/10.1021/ja01524a073*
3. Szyling, J., Franczyk, A., Pawluc, P., Marciniec, B., Walkowiak, J. (2017) A stereoselective synthesis of (*E*)-or (*Z*)-β-arylvinyl halides via a borylative coupling/halodeborylation protocol, *Org Biomol Chem* 15, 3207−3215. *https://doi.org/10.1039/C7OB00054E*
4. Lennox, A. J., Lloyd-Jones, G. C. (2014) Selection of boron reagents for Suzuki−Miyaura coupling, *Chem Soc Rev* 43, 412−443. *https://doi.org/10.1039/C3CS60197H*
5. Miyaura, N., Suzuki, A. (1995) Palladium-catalyzed cross-coupling reactions of organoboron compounds, *Chem Rev* 95, 2457−2483. *https://doi.org/10.1021/cr00039a007*
6. Fagnou, K., Lautens, M. (2003) Rhodium-catalyzed carbon−carbon bond-forming reactions of organometallic compounds, *Chem Rev* 103, 169–196. *https://doi.org/10.1021/cr020007u*

7. Rau, H. H., Werner, N. S. (2018) Stereocontrolled synthesis of (*E*)-stilbene derivatives by palladium-catalyzed Suzuki-Miyaura cross-coupling reaction, *Bioorg Med Chem Lett* 28, 2693-2696. *https://doi.org/10.1016/j.bmcl.2018.04.004*
8. Clausen, F., Kischkewitz, M., Bergander, K., Studer, A. (2019) Catalytic protodeboronation of pinacol boronic esters: formal *anti*-Markovnikov hydromethylation of alkenes, *Chem Sci* 10, 6210–6214. *https://doi.org/10.1039/c9sc02067e*
9. Liu, C., Li, X., Wu, Y. (2015) Base-promoted silver catalyzed protodeboronation of arylboronic acids and esters, *RSC Adv* 5, 15354–15358. *https://doi.org/10.1039/c4ra16323k*
10. Yamamoto, Y., Takizawa, M., Yu, X.-Q., Miyaura, N. (2008) Cyclic triolborates: air- and water-stable ate complexes of organoboronic acids, *Angew Chem Int Ed* 47, 928–931. *https://doi.org/10.1002/anie.200704162*
11. Sakae, R. Hirano, K., Satoh, T., Miura, M. (2013) Formal *anti*-Markovnikov hydroamination of terminal aryl alkynes with pinacolborane and hydroxylamines via Zr/Cu sequential catalysis, *Chem Lett* 42, 1128–1130. *https://doi.org/10.1246/cl.130485*
12. Vantourout, J. C., Law, R. P., Isidro-Llobet, A., Atkinson, S. J., Watson, A. J. B. (2016) Chan-Evans-Lam amination of boronic acids pinacol (Bpin) esters: Overcoming the Aryl Amine Problem, *J Org Chem* 81, 3942–3950. *https://doi.org/10.1021/acs.joc.6b00466*
13. Sueki, S., Kuninobu, Y. (2013) Copper-catalyzed *N*- and *O*-alkylation of amines and phenols using alkylborane reagents, *Org Lett* 15, 1544–1547. *https://doi.org/10.1021/ol400323z*
14. Feng, C., Loh, T.-P. (2014) Rhodium(III)-catalyzed cross-coupling of alkenylboronic acids and *N*-pivaloyloxylamides, *Org Lett* 16, 3444–3447. *https://doi.org/10.1021/ol501309e*
15. Shade, R. E., Hyde, A. M., Olsen, J.-C., Merlic, C. A. (2010) Copper-promoted coupling of vinyl boronates and alcohols: a mild synthesis of allyl vinyl ethers, *J Am Chem Soc* 132, 1202–1203. *https://doi.org/10.1021/ja907982w*
16. Marcum, J. S., McGarry, K. A., Ferber, C. J., Clark, T. B. (2016) Synthesis of biaryl ethers by the copper-catalyzed Chan-Evans-Lam etherification from benzylic amine boronate esters, *J Org Chem* 81, 7963–7969. *https://doi.org/10.1021/acs.joc.6b01254*
17. Chan, D. G., Winternheimer, D. J., Merlic, C. A. Enol silyl ethers via copper(II)-catalyzed C–O bond formation, *Org Lett* 13, 2778–2781. *https://doi.org/10.1021/ol2009297*
18. Kabalka, G. W., Guchhait, S. K. (2003) Synthesis of (*E*)- and (*Z*)-alkenylphosphonates using vinylboronates, *Org Lett* 5, 729–731. *https://doi.org/10.1021/ol027515a*
19. Zhuang, R., Xu, J., Cai, Z., Tang, G., Fang, M., Zhao, Y. (2011) Copper-catalyzed C–P bond construction via direct coupling of phenylboronic acids with *H*-phosphonate diesters, *Org Lett* 13, 2110–2113. *https://doi.org/10.1021/ol200465z*
20. Kanemoto, K., Yoshida, S., Hosoya, T. (2018) Modified conditions for copper-catalyzed *ipso*-thiolation of arylboronic acid esters with thiosulfonates, *Chem Lett* 47, 85–88. *https://doi.org/10.1246/cl.170907*
21. Xu, H.-J., Zhao, Y.-Q., Feng, T., Feng, Y.-S. (2012) Chan-Lam-type *S*-arylation of thiols with boronic acids at room temperature, *J Org Chem* 77, 2878–2884. *https://doi.org/*10.1021/jo300100x
22. Scott, H. K., Aggarwal, V. K. (2011) Highly enantioselective synthesis of tertiary boronic esters and their stereospecific conversion to other functional groups and quaternary stereocentres, *Chem Eur J* 17, 13124–13132. *https://doi.org/10.1002/chem.201102581*
23. Brown, H. C. (1961) Hydroboration–a powerful synthetic tool, *Tetrahedron* 12, 117–138. *https://doi.org/10.1016/0040-4020(61)80107-5*
24. Woods, W. G., Strong, P. L., (1966) 4,4,6-Trimethyl-1,3,2-dioxaborinane. A stable dialkoxyborane, *J Am Chem Soc* 88, 4667–4671. *https://doi.org/10.1021/ja00972a027*
25. Irvine, G. J., Lesley, M. J. G., Marder, T. B., Norman, N. C., Rice, C. R., Robins, E. G., Roper, W. R., Whittell, G. R., Wright L. J., (1998) Transition metal−boryl compounds: synthesis, reactivity, and structure, *Chem Rev* 98, 2685–2722. *https://doi.org/10.1021/CR9500085*
26. Beletskaya, I., Pelter, A., (1997) Hydroborations catalyzed by transition metal complexes, *Tetrahedron* 53, 4957–5026. *https://doi.org/10.1016/S0040-4020(97)00001-X*
27. Bauer, I., Knölker, H.-J. (2015) Iron catalysis in organic synthesis, *Chem Rev* 115, 3170–3387. *https://doi.org/10.1021/cr500425u*
28. Gorgas, N., Alves, L. G., Stö, B., Martins, A. M., Veiros, L. F., Kirchner, K. (2017) Stable, yet highly reactive nonclassical iron(II) polyhydride pincer complexes: Z-selective dimerization and hydroboration of terminal alkynes, *J Am Chem Soc* 139, 8130–8133. *https://doi.org/10.1021/jacs.7b05051*
29. Mandal, S., Verma, P. K., Geetharani, K. (2018) Lewis acid catalysis: regioselective hydroboration of alkynes and alkenes promoted by scandium triflate, *Chem Commun* 54, 13690–13693. *https://doi.org/10.1039/C8CC08361D*
30. Mamidala, R., Pandey, V. K., Rit, A. (2019) $AgSbF_6$-catalyzed *anti*-Markovnikov hydroboration of terminal alkynes, *Chem Commun* 55, 989–992. *https://doi.org/10.1039/C8CC07499B*
31. Jang, W. J., Kang, B.-N., Lee, J. H., Choi, Y. M., Kim, C.-H., Yun, J. (2019) NHC-copper-thiophene-2-caroxylate complex for the hydroboration of terminal alkynes, *Org Biomol Chem* 17, 5249–5252. *https://doi.org/10.1039/C9OB00839J*
32. Wang, Y., Guan, R., Sivaguru, P., Cong, X., Bi, X. (2019) Silver-catalyzed *anti*-Markovnikov hydroboration of C–C multiple bonds, *Org Lett* 21, 4035–4038. *https://doi.org/10.1021/acs.orglett.9b01217*

33. Hill, M. S., Liptrot, D. J., Weetman, C. (2016) Alkaline earths as main group reagents in molecular catalysis, *Chem Soc Rev* 45, 972–988. *https://doi.org/10.1039/C5CS00880H*

34. Magre, M., Maity, B., Falconnet, A., Cavallo, L., Rueping, M. (2019) Magnesium-catalyzed hydroboration of terminal and internal alkynes, *Angew Chem Int Ed* 58, 7025–7029. *https://doi.org/10.1002/anie.201902188*

35. Nikonov, G. I. (2017) New tricks for an old dog: aluminum compounds as catalysts in reduction chemistry, *ACS Catal* 7, 7257–7266. *https://doi.org/10.1021/acscatal.7b02460*

36. Bismuto, A., Cowley, M. J., Thomas, S. P. (2018) Aluminum-catalyzed hydroboration of alkenes, *ACS Catal* 8, 2001–2005. *https://doi.org/10.1021/acscatal.7b04279*

37. Harinath, A., Banerjee, I., Bhattacharjee, J., Panda, T. K. (2019) Aluminum complex-catalyzed hydroboration of alkenes and alkynes, *New J Chem* 43, 10531–10536. *https://doi.org/10.1039/c9nj01859j*

38. Wu, Y., Shan, C., Ying, J., Su, J., Zhu, J., Liu, L. L., Zhao, Y. (2017) Catalytic hydroboration of aldehydes, ketones, alkynes and alkenes initiated by NaOH, *Green Chemistry* 19, 4169–4175. *https://doi.org/10.1039/C7GC01632H*

39. Ma, D. H., Jaladi, A. K., Lee, J. H., Kim, T. S., Shin, W. K., Hwang, H., An, D. K. (2019) Catalytic hydroboration of aldehydes, ketones, and alkenes using potassium carbonate: a small key to big transformation *ACS Omega* 4, 15893–15903. *https://doi.org/10.1021/acsomega.9b01877*

40. Garrett, C. E., Fu, G. C. (1996) Hydroboration of olefins with catecholborane at room temperature in the presence of *N*,*N*-dimethylacetamide, *Org Chem* 61, 3224–3225. *https://doi.org/10.1021/JO960386P*

41. Ho, H. E., Asao, N., Yamamoto, Y., Jin, T. (2014) Carboxylic acid-catalyzed highly efficient and selective hydroboration of alkynes with pinacolborane, *Org Lett* 16, 4670–4673. *https://doi.org/10.1021/ol502285s*

42. Hoyt, C. B., Sarazen, M. L., Jones, C. W. (2019) Hydroboration of substituted alkynes using a solid polymeric carboxylic acid catalyst, *J Catal* 369, 493–500. *https://doi.org/10.1016/j.jcat.2018.11.021*

43. Suseela, Y., Prasad, A. S., Periasamy, M., (1990) Catalytic effect of a BH_3:*N*,*N*-diethylaniline complex in the formation of alkenyl catecholboranes from alk-1-ynes and catecholborane, *J Chem Soc, Chem Commun* 446–447. *https://doi.org/10.1039/C39900000446*

44. Hoshi, M., Shirakawa, K., Arase, A. (2004) Preparation of (*E*)-1-Alkenylboronic Acid Pinacol Esters via Transfer of Alkenyl Group from Boron to Boron, *Synthesis* 11, 1814–1820. *https://doi.org/10.1055/s-2004-829165*

45. Arase, A., Hoshi, M., Mijin, A., Nishi, K. (2006) Dialkylborane-catalyzed hydroboration of alkynes with 1,3,2-benzodioxaborole in tetrahydrofurane, *Synth Commun* 25, 1957–1962. *https://doi.org/10.1080/00397919508015872*

46. Anj, N. W., Buettner, C. S., Docherty, S., Bismuto, A., Carney, J. R., Docherty, J. H., Cowley, M. J., Thomas, S. P. (2018) Borane-Catalyzed Hydroboration of Alkynes and Alkenes, *Synthesis* 50, 803–808. *https://doi.org/10.1055/s-0036-1591719*

47. Yin, Q., Kemper, S., Klare, H. F. T., Oestreich, M. (2016) Boron Lewis acid-catalyzed hydroboration of alkenes with pinacolborane: BAr^{F}_3 does what $B(C_6F_5)_3$ cannot do, *Chem Eur J* 22, 13840–13844. *http://dx.doi.org/10.1002/chem.201603466*

48. Carden, J. L., Gierlichs, L. J., Wass, D. F., Browne, D. L., Melen, R. L. (2019) Unlocking the catalytic potential of tris(3,4,5-trifluorophenyl)borane with microwave irradiation, *Chem Commun* 55, 318–321. *http://dx.doi.org/10.1039/C8CC09459D*

49. 9-BBN was used as a 0.5 M solution in THF. The final reaction concentration was 2.5 M.

50. Hitosugi, S., Tanimoto, D., Nakanishi, W., Isobe, H. (2012) A facile chromatographic method for purification of pinacol boronic esters, *Chem Lett* 41, 972–973. *https://doi.org/10.1246/cl.2012.972*

51. Peng, A.-Y., Chen, B.-T., Chen, P.-J. (2013) An efficient synthesis of 2-(1-(*E*)-alkenyl)phenylphosphonates via Suzuki reaction of aryl nonaflates with (*E*)-1-alkenylboronates, *J Fluorine Chem* 151, 58–62. *https://doi.org/10.1016/j.jfluchem.2013.03.015*

52. Fairlamb, I. J. S., Marrison, L. R., Dickinson, J. M., Lu, F.-J., Schimdt, J.P. (2004) 2-Pyrones possessing antimicrobial and cytotoxic activities, *Bioorgan Med Chem* 12, 4285–4299. *https://doi.org/10.1016/j.bmc.2004.01.051*

ABOUT THE STUDENT AUTHORS

Garett Ruesch graduated Summa Cum Laude from Southern Utah University in 2020 with an ACS-certified B.S. degree in chemistry with an emphasis in professional chemistry and minors in Spanish and mathematics. While at SUU, Garett was a two-time recipient of the Walter Maxwell Gibson Research Fellowship, and was awarded the 2019 ACS Undergraduate Award in Physical Chemistry and 2020 ACS Division of Organic Chemistry Outstanding, Senior Chemistry Student at SUU. He currently pursues a Ph.D. degree in chemistry at the University of California, Los Angeles and ultimately a professorship.

Sydney Rowley plans to graduate from Southern Utah University in the spring of 2021 with an ACS-certified B.S. degree in chemistry with an emphasis in professional chemistry and a minor in mathematics. Sydney has received the Walter Maxwell Gibson Research Fellowship and the L. S. and Aline W. Skaggs Research Grant. After graduation, she plans on pursuing a Ph.D. degree in chemistry and a career in the pharmaceutical industry.

Marcus Mifflin graduated Summa Cum Laude from Southern Utah University in 2019 with an ACS-certified B.S. degree in chemistry with an emphasis in professional chemistry and minors in mathematics and physics. While at SUU, Marcus was the

recipient of the Walter Maxwell Gibson Research Fellowship, and he received the Outstanding Research Award in the Department of Physical Science. He currently pursues a Ph.D. degree in chemistry at the University of Utah and ultimately a professorship.

PRESS SUMMARY

Boron containing organic compounds are useful reagents in the synthesis of important carbon-containing medicines, materials, and fine chemicals. The alkenylboronic acid pinacol esters formed from hydroboration reaction of alkynes with pinacolborane are stable, easy to handle, and useful in many synthetic transformations. However, pinacolborane lacks the reactivity necessary to undergo facile hydroboration reaction with terminal aromatic alkynes. It was discovered that 9-borobicyclo[3.3.1]nonane (9-BBN) can be used to catalyze the hydroboration reaction of terminal aromatic alkynes with pinacolborane. The parameters of the experimental procedure were evaluated and an optimal set of reaction conditions were found. This work provides synthetic chemists with a method to prepare these important compounds.

Synthetic Biology Bicistronic Designs Support Gene Expression Equally Well *in vitro* and *in vivo*

Owen Koucky[a], Jacob Wagner[b], Sofia Aguilera[b], Benjamin Bashaw[b], Queena Chen[a], Anthony Eckdahl[b], Elise Edman[c], Paul Gomez[a], Nick Hanlan[b], Nick Kempf[b], Devin Mattoon[d], Sam McKlin[a], Christopher Mazariegos[a] , Alex Morehead[d], Shi Qing Ong[b], Andy Peterson[c], Maria Rojas[a], Kyla Roland[a], Kaitlyn Schildknecht[d], Haley Seligmann[c], Kaden Slater[d], Ali Tauchen[b], Raechel Tittor[b], Tatianna Travieso[a], Dannie Urban[d], Caroline Willis[a], John Zhou[a], Nicole L. Snyder[e], Laurie J. Heyer[c], Jeffrey L. Poet[d], Todd T. Eckdahl[b], & A. Malcolm Campbell[a]*

[a]*Department of Biology, Davidson College, Davidson, NC*
[b]*Department of Biology, Missouri Western State University, St. Joseph, MO*
[c]*Department of Mathematics and Computer Science, Davidson College, Davidson, NC*
[d]*Department of Computer Science, Math, and Physics, Missouri Western State University, St. Joseph, MO*
[e]*Department of Chemistry, Davidson College, Davidson, NC*

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Students: owkoucky@davidson.edu, jwagner3@missouriwestern.edu, saguileratovar@missouriwestern.edu, bbashaw@missouriwestern.edu, quchen@davidson.edu, aneckdahl@davidson.edu, eledman@davidson.edu, pagomez@davidson.edu, nhanlan@missouriwestern.edu, nkempf1@missouriwestern.edu, dmattoon@missouriwestern.edu, samcklin@davidson.edu, chmazariegos@davidson.edu, amorehead1@missouriwestern.edu, song@missouriwestern.edu, apeterson5@missouriwestern.edu, marojas@davidson.edu, kyroland@davidson.edu, kschildknecht@missouriwestern.edu, haseligmann@davidson.edu, kslater3@missouriwestern.edu, atauchen1@missouriwestern.edu, rtittor@missouriwestern.edu, tatravieso@davidson.edu, durban1@missouriwestern.edu, cawillis@davidson.edu, jzhou@davidson.edu
*Mentors: nisnyder@davidson.edu, laheyer@davidson.edu, poet@missouriwestern.edu, eckdahl@missouriwestern.edu, macampbell@davidson.edu**

ABSTRACT
Synthetic biology integrates molecular biology tools and an engineering mindset to address challenges in medicine, agriculture, bioremediation, and biomanufacturing. A persistent problem in synthetic biology has been designing genetic circuits that produce predictable levels of protein. In 2013, Mutalik and colleagues developed bicistronic designs (BCDs) that make protein production more predicable in bacterial cells (*in vivo*). With the growing interest in producing proteins outside of cells (*in vitro*), we wanted to know if BCDs would work as predictably in cell-free protein synthesis (CFPS) as they do in *E. coli* cells. We tested 20 BCDs in CFPS and found they performed very similarly *in vitro* and *in vivo*. As a step toward developing methods for protein production in artificial cells, we also tested 3 BCDs inside nanoliter-scaled microfluidic droplets. The BCDs worked well in the microfluidic droplets, but their relative protein production levels were not as predictable as expected. These results suggest that the conditions under which gene expression happens in droplets result in a different relationship between genetic control elements such as BCDs and protein production than exists in batch CFPS or in cells.

KEYWORDS
Bicistronic Design; Synthetic Biology; Cell-Free Protein Synthesis; Microfluidics

INTRODUCTION

Synthetic biology is a new field that integrates molecular biology, engineering, chemistry, mathematics and computer science.[1] Synthetic biologists have invented many useful devices that produce medicines[2,3,4], improve agriculture,[5] achieve bioremediation[6] and make biofuels or chemical commodities through biomanufacturing.[7] Despite these successes, a persistent challenge in synthetic biology has been consistent and reliable protein production. To address this challenge, a team of synthetic biologists invented bicistronic designs (BCDs) that produce predictable levels of protein in *E. coli*.[8] BCDs are encoded in DNA but function as part of mRNA. A BCD consists of two segments of DNA called cistrons that encode polypeptides. The first cistron begins with a ribosomal binding site (RBS) followed by a start codon that marks the beginning of a coding segment for a non-functional leader polypeptide of 16 amino acids. The stop codon for the coding segment overlaps by one base pair with the start codon for the second cistron (see **Figure 4A**). Upstream of this start codon, and within the 48 bases encoding the leader polypeptide, is an RBS element that directs the production of a protein from the second cistron. This second RBS was the focus of the research by Mutalik and colleagues. They varied the strength of the second RBS and the amount of protein translated varied accordingly. The authors speculated that during translation of the first polypeptide, the large ribosome and its helicase activity eliminated folded mRNA structures formed by base pairing between the second RBS and the beginning of the coding sequence in the second

cistron. Thus, the second RBS was allowed to base pair with the 16S rRNA of the ribosome without interference from mRNA folding and a predictable amount of two different reporter proteins was produced with each of the RBSs tested.[8]

Although cells are very good at producing proteins, cells often cannot produce robust amounts of orthogonal proteins and sometimes cannot produce them at all.[9] For example, *E. coli* will not produce antimicrobial proteins that kill bacteria.[10] Therefore, synthetic biologists sometimes switch from *in vivo* production of proteins to *in vitro* production.[11,12,13] The purpose of our research was to determine if the BCDs described by Mutalik and colleagues would work as predictably *in vitro* as they do *in vivo*. To conduct this work, we chose to develop an affordable cell-free protein synthesis (CFPS) protocol instead of purchasing expensive pre-made mixtures. Once we had a functional CFPS protocol, we tested 20 of the BCDs made by Mutalik and colleagues during CFPS. As a step toward developing methods for protein production in artificial cells, we also compared protein production directed by 3 BCDs in CFPS droplets to batch CFPS reactions with the same 3 BCDs.

METHODS AND PROCEDURES

The protocols for CFPS and microfluidics are too extensive to give all the details in this paper, so we have compiled all methods into the appendix. However, we summarize the main steps below. Much of our method has been adapted from multiple protocols.[14,15,16,17]

Cell-free Protein Synthesis for Batch Reactions and Protein Quantification

E. coli encoding the T7 RNA polymerase (BL21 (DE3) *E. coli* cells; NEB # C2527H) were grown in LB broth supplemented with IPTG to induce T7 RNA polymerase expression. Cells were grown overnight and harvested by centrifugation. Cells were lysed using an AVESTIN EmiulsiFlex B-15 high-pressure homogenizer, also known as a French press. Cytoplasmic lysate was aliquoted and stored at -80 °C for later use. The CFPS reaction conditions were as follows: 55 mM PIPES-KOH (pH 7.0); 14 mM magnesium acetate; 50 mM potassium acetate; 155 mM ammonium acetate; 1.5% PEG-6000; 40 mM 3-phosphoglycerate; amino acid cocktail with all 20 naturally occurring protein amino acids at 2.5 mM each; 1.2 mM ATP; 1.0 mM GTP; 0.8 mM UTP; DNA template at 10 ng/μL; and 2.5 μL *E. coli* cell lysate for a final reaction volume of 25 μL. We have an Excel template file we use to make the reaction mixtures and this tool is part of the appendix.

Once the ingredients are mixed, we placed the 25 μL aliquots into a 384-well plate and incubated it at the desired temperature for variable lengths of time, as indicated. Plates containing CFPS were scanned at the appropriate wavelength for GFP (excitation 485 nm, emission 515 nm) to quantify the relative fluorescence. The fluorometer was programmed to measure the wells at regular intervals and incubate the plate at the desired temperature between measurements.

Cell-free Protein Synthesis in Microfluidic Droplets

We used the OB1 MK3 Microfluidic Flow Control System from Elveflow to generate droplets for CFPS. CFPS reactions for use in microfluidic droplets were prepared as described above for *in vitro* batch reactions. For each reaction, the aqueous (dispersive phase) reservoir for microfluidic droplet generation was filled with 30 μL of this CFPS mixture, 10 μL of nuclease-free water, and 10 μL containing 500 ng of plasmid DNA for BCD9, BCD19, or BCD24 gene expression constructs (or 10 μL of water for the no DNA negative control). The aqueous reservoir was vortexed for about 5 seconds. For each reaction, the oil (continuous phase) reservoir was filled with 50 μL droplet stabilization oil (Droplet Genomics cat. DG-DSO-20). Both the dispersive and continuous channels were set on the OB1 MK3 to 600 mbar and droplets were collected in a 1.5 ml tube. For each reaction, a volume of 50 μL of droplets was loaded into a 384 well microtiter plate for use in the fluorometer. Positive control batch reactions with 100% CFPS mixture and 75% CFPS mixture were also loaded into the plate. The fluorometer was set to 37 °C and GFP expression was measured after 3 hours using excitation and emission wavelengths of 485 nm and 528 nm respectively.

RESULTS

Producing the *E. coli* cell lysate went smoothly but optimizing CFPS took considerable effort (**Figure 1**). The first challenge was making the amino acid cocktail without precipitating any of the components. The original protocol called for neutralizing the acidic pH of the mixtures, but the only way we could get CFPS to work was when we did not adjust the pH of the amino acid solution (**Figure 1A**).[18] We tested a range of temperatures and found that 30 °C was optimal, but we used 37 °C for potential applications using different proteins. The literature reported the use of a range of PEG-6000 concentrations,[16] but we chose 1.5% as a compromise between maximum protein production (**Figure 1C**) and fastest induction time (**Figure 1D**), defined as the time at which GFP production was twice the background level.

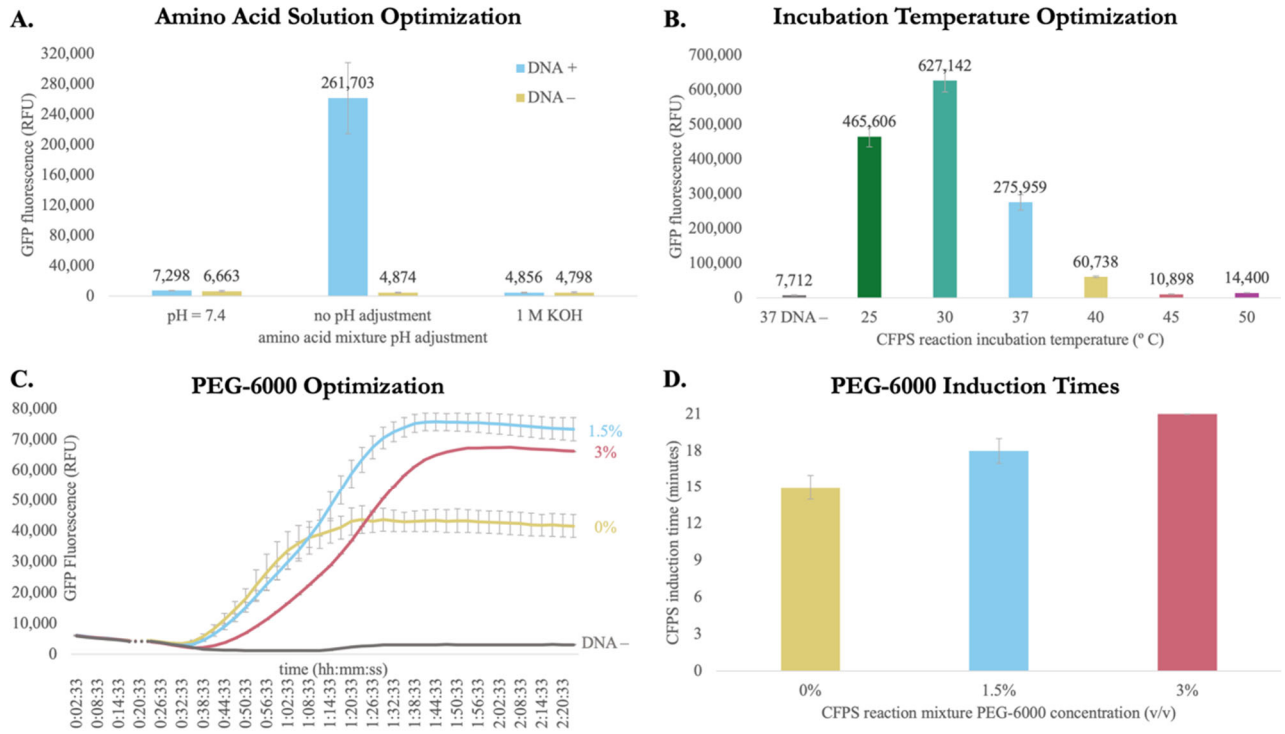

Figure 1. Characterization of CFPS. A. Varying preparations of the 20-amino acid solution for use in the CFPS reaction mixture by adjusting the pH to 7.4, omitting the pH adjustment step, or raising the pH by increasing KOH concentration to 1 M resulted in variable CFPS performance. B. Characterization of CFPS performance across a range of incubation temperatures. C. CFPS reactions with varying concentrations of PEG-6000 (% v/v) affected CFPS output. D. CFPS induction time also varied with PEG-6000 concentrations. CFPS performance verification experiments used the March lysate while PEG-6000 optimization experiments used the July lysate. Error bars represent ± 1 S.E (n=9).

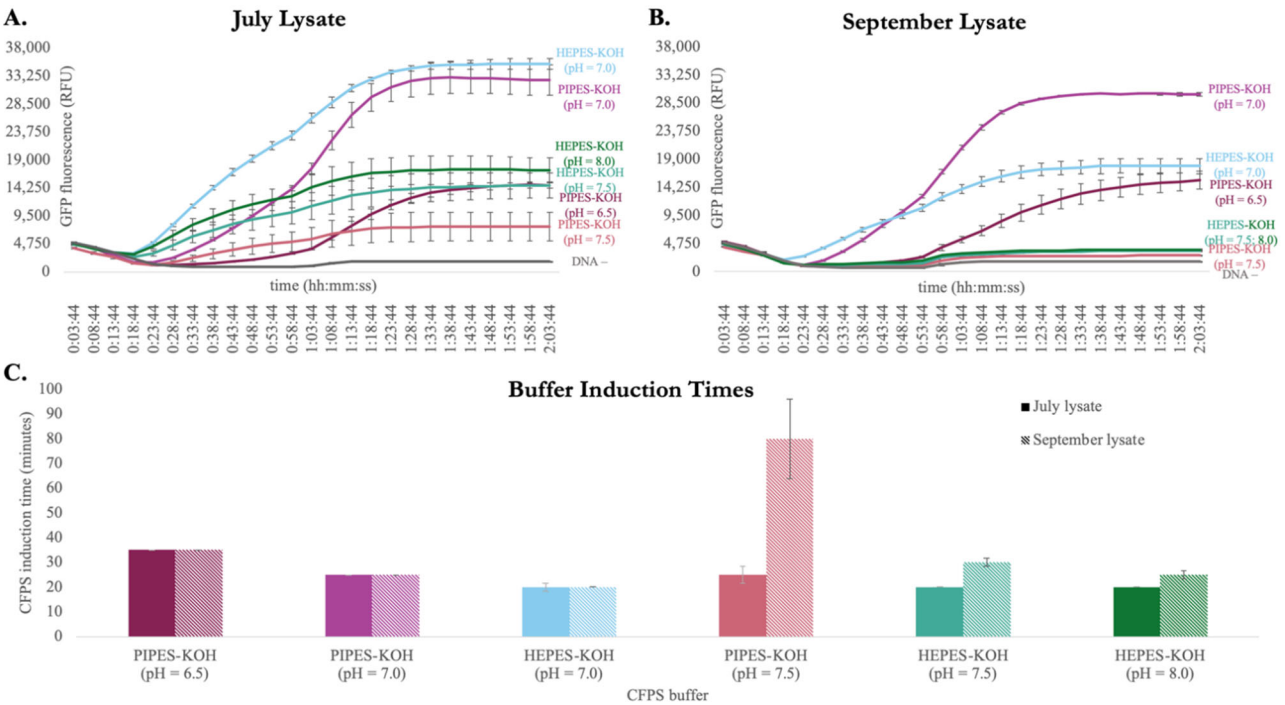

Figure 2. Comparison of cell lysate preparations. A, B. July and September lysates were used in CFPS reactions with six buffers that varied from pH 6.5 to 8.0: PIPES-KOH (pH = 6.5); PIPES-KOH (pH = 7.0); HEPES-KOH (pH = 7.0); PIPES-KOH (pH = 7.5); HEPES-KOH (pH = 7.5); and HEPES-KOH (pH = 8.0). C. CFPS induction time compared across the six buffers and two lysate treatments. Error bars represent ± 1 S.E (n=9).

An unexpected complication was optimizing the pH of the CFPS reaction mixture. We found that each lysate preparation had its own optimal buffer and optimal pH (**Figure 2**). For example, the lysate produced in July worked best in HEPES-KOH pH 7.0 whereas the September lysate worked best in PIPES-KOH pH 7.0. We do not have an explanation for these observations. Induction times for GFP production also varied depending on the buffer and pH employed. For most experiments, we chose PIPES-KOH pH 7.0 as our standard reaction condition since this buffer was incorporated in functional CFPS across multiple lysate preparations.

We were curious about why all the time-course fluorescence readings in **Figures 1 and 2** began with a decline in fluorescence. We hypothesized that CFPS did not commence immediately and that some chemical reaction that altered the pH had to take place first. We suspected the pH might need to be closer to 7.4, so we withheld the DNA for variable amounts of time from reactions incubated at 37 ° C and measured the pH during the entire experiment (**Figure 3**). If the CFPS reaction is allowed to pre-incubate for 15 minutes, then the final protein output is substantially greater. It was striking to us that whether the reaction contained DNA or not, the pH gradually fell from about 7.9 to near 7.0. The decrease in fluorescence between pH measurements was shown to be temperature-dependent because it diminished as we reduced the incubation temperature within the fluorometer. As described in the literature, when we added maltose to a final concentration of 12 mM after 2 hours of reaction, we doubled the final protein output.[18]

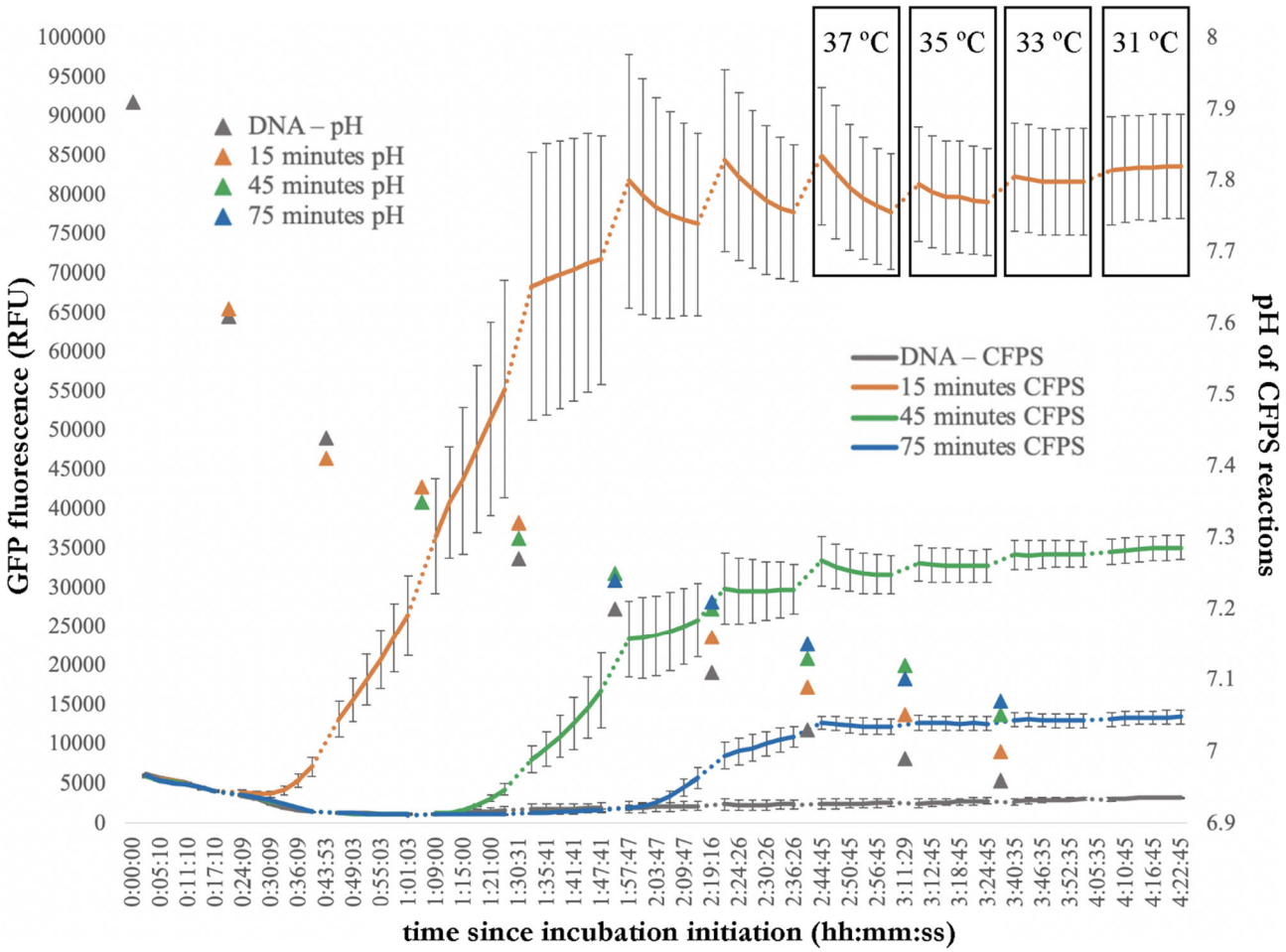

Figure 3. Analysis of CFPS GFP production and pH changes. CFPS reaction mixture pre-DNA incubation times are indicated in the legend. Dotted sections of the graph represent the duration of measurement periods to account for the time the CFPS reactions spent out of the plate reader during pH sampling. CFPS employed the July lysate. Error bars represent ± 1 S.E (n=9).

Once we had optimized CFPS, we tested 20 of the BCDs from Mutalik and colleagues (**Figure 4**).[8] We ranked the GFP output from highest (BCD19) to lowest (BCD22). When we compared our GFP fluorescence levels from **Figure 4** to those from the Mutalik paper with Spearman's Rank Order Correlation, we found a high degree of correlation (r = 0.88). From the data in Figure 4B and the high degree of concordance with Mutalik's data, we conclude that the BCDs first developed for *in vivo* use work similarly *in vitro*. This is the first publication showing BCDs function *in vitro*.

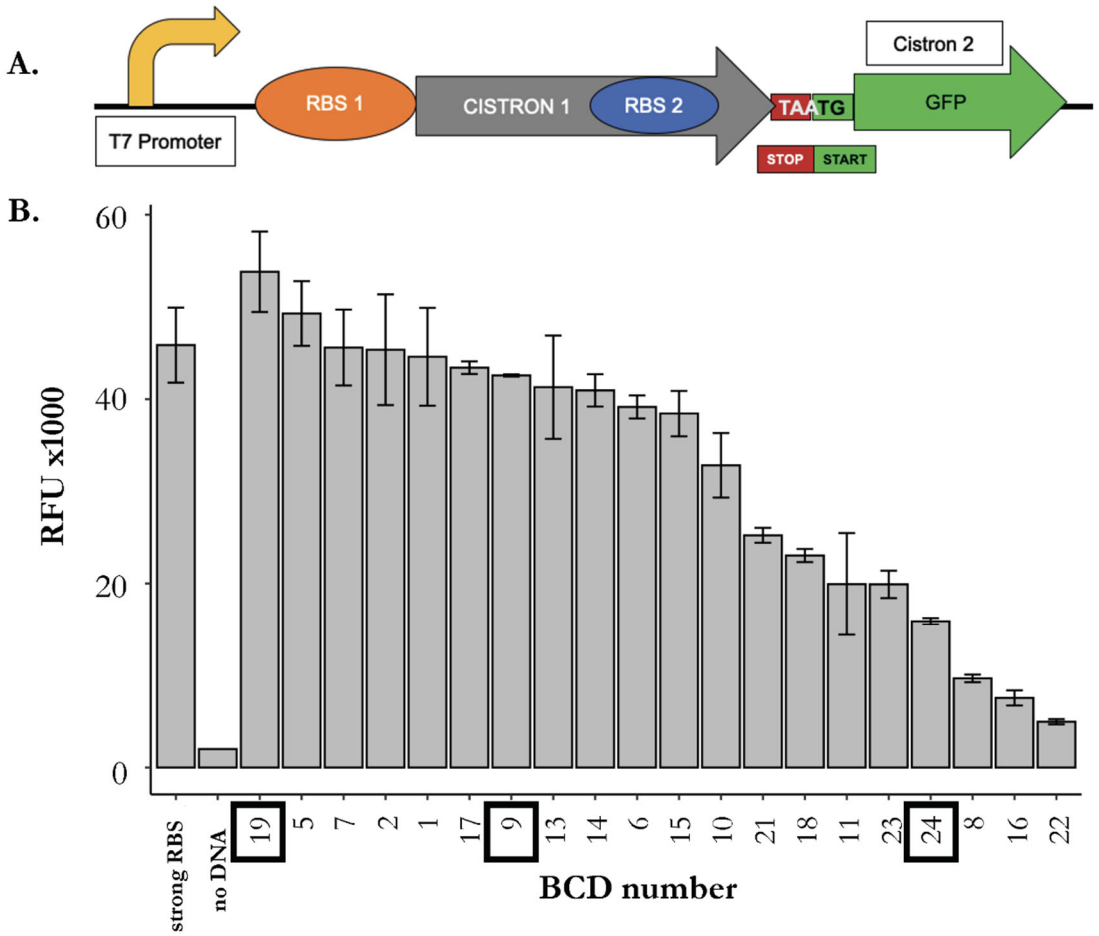

Figure 4. CFPS using bicistronic designs A. Diagram of BCD components as part of a gene expression cassette that also includes a T7 promoter and a GFP reporter gene (not drawn to scale). B. Fluorescence output after three hours of CFPS reactions with 20 different BCDs, a strong RBS positive control, and a no DNA negative control. Error bars represent ± 1 S.E (n=9). The 3 boxed BCDs were used for droplet CFPS in **Figure 5**.

After establishing the functionality of BCDs in CFPS batch reactions, we explored their potential for use in nanoliter-scale microfluidic droplets (**Figure 5A**). To realize our goal of using microfluidic droplets for metabolic engineering will require further exploration of the function of gene regulatory elements such as BCDs in nanoscale droplets. Through experimentation, we discovered the normal CFPS reaction mixture was too viscous to produce reliable droplets, so we diluted the droplet reaction mixture with water to 75% of the concentration of CFPS batch reactions (**Figure 5B**). We tested three of the BCDs (19, 9 and 24) from **Figure 4** that represent a range of strengths. We also compared 75% reaction mixtures in batch reactions and droplet CFPS. The 100% CFPS batch reactions performed as we expected, though the 75% pooled CFPS varied a bit more than we had predicted. However, we were surprised to see that 75% concentration CFPS within the droplets outperformed the same concentration in pooled CFPS. We observed that the measured collection of droplets was about 75% oil by volume and 25% aqueous CFPS by volume. Therefore, the overall observed output of GFP from CFPS in droplets is actually 4-fold higher than is represented in the batch results of **Figure 4**. We offer two possible explanations for this result. One is that the reflective nature of the oil and the geometry of the droplets enhances GFP detection, and the other is that CFPS happened more efficiently in droplet CFPS than in CFPS batch reactions. We also found that in droplet CFPS, the relative fluorescence outputs from BCD19 and 9 are reversed compared to the results *in vivo* or in CFPS batch reactions *in vitro*. These results suggest that the reaction conditions under which gene expression happens in droplets result in a different relationship between BCDs and protein production than exists in batch CFPS or in cells. Due to the complexity of microfluidic droplet formation, there also could be other reasons for these unexpected results.

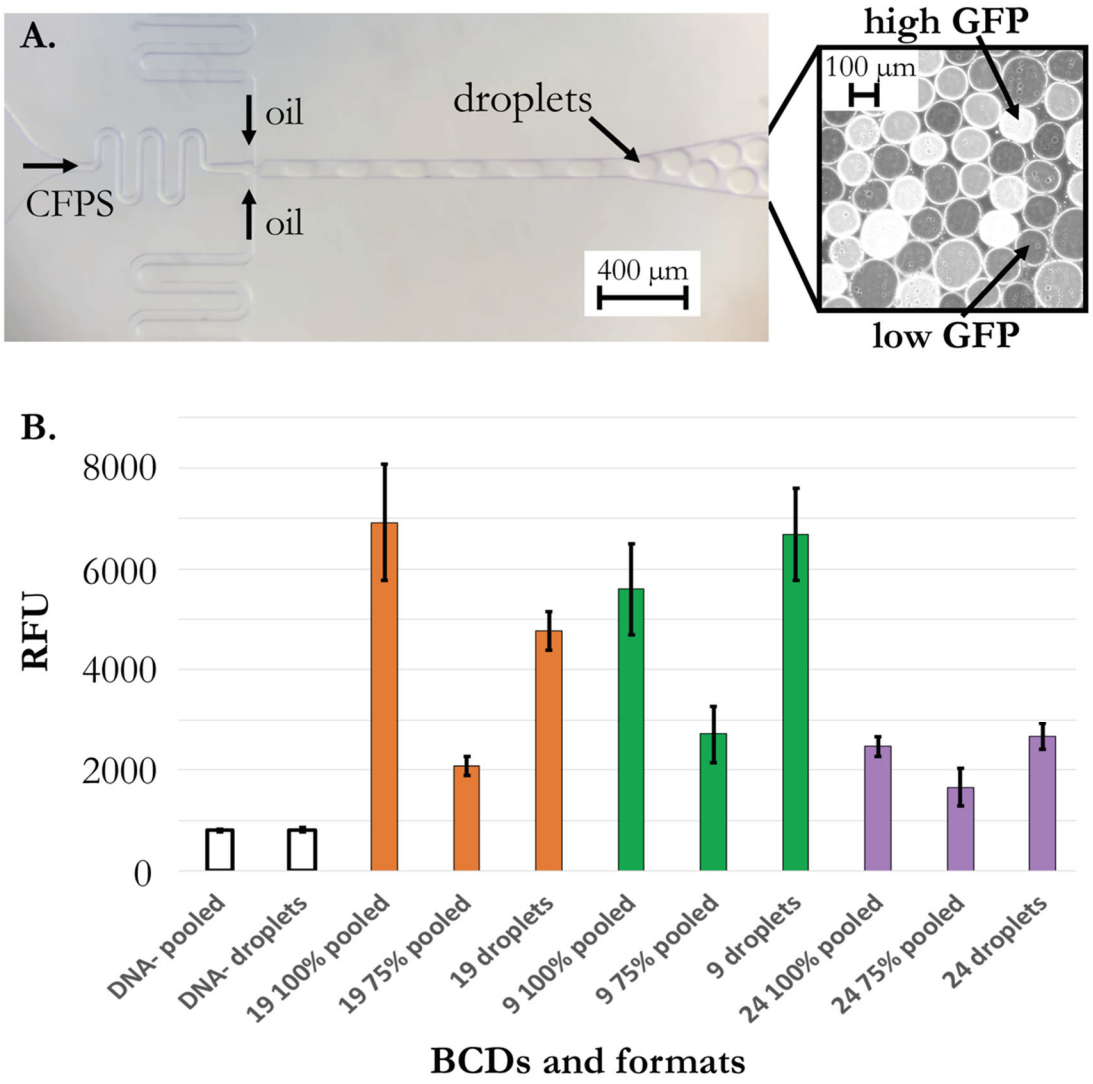

Figure 5. CFPS using 3 BCDs in microfluidic droplets. A. The left micrograph shows a perpendicular junction PDMS droplet-generation microchip with labels that indicate the flow of the aqueous CFPS dispersive phase, the oil continuous phase, and droplets immersed in oil. The right micrograph was taken on a fluorescence microscope and shows various levels of GFP produced by CFPS. B. Fluorescence output after three hours with BCD19, BCD9, and BCD24 using 100% CFPS solutions in pooled reactions, 75% CFPS solutions in pooled reactions, 75% CFPS solutions in populations of microfluidic droplets, and no DNA (DNA-) negative controls. Error bars represent ± 1 S.E (n=9).

CONCLUSIONS

In this paper, we describe a less expensive approach to CFPS using cell lysate produced locally, as spelled out in the detailed protocols of the appendix (**Figures 1 – 3**). The goal of this research was to determine if BCDs performed similarly *in vitro* and *in vivo*. As shown by the results presented in **Figure 4** and **Figure 5**, our Spearman's Rank Order Correlation calculations show the BCDs function equivalently in pooled CFPS and droplet CFPS. Furthermore, we found that GFP detection is amplified in droplets, either because the reflection of light enhanced the GFP signal detected in a droplet or oil mixture or CFPS in droplets was more efficient. Although the BCDs worked well in the microfluidic droplets, their relative expression levels were not as consistent as expected. These complex results are not surprising given that Figure 5 is the first published example of BCD gene expression in microfluidic droplets. Additional research will be required to determine if CFPS with BCDs is more efficient or if

GFP light reflection produces enhanced light detection in the absence of more protein production. Furthermore, more research will be required to determine the source of droplet variability for GFP production.

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REFERENCES
1. Campbell, A. M. (2005) Meeting Report: Synthetic Biology Jamboree for Undergraduates, *Cell Biology Education* 4(1), 19–23. *doi:10.1187/cbe.04-11-0047*
2. Denby, C. M., Li, R. A., Vu, V. T., Costello, A., Lin, W., Chan, L. J. G., Williams, J., Donaldson, B., Bamforth, C. W., Petzold, C. J., Scheller, H. V., Martin, H. G., Keasling, J. D. (2018) Industrial brewing yeast engineered for the production of primary flavor determinants in hopped beer, *Nat Commun* 9, 965. *https://doi.org/10.1038/s41467-018-03293-x*
3. Ro, D.-K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Ho, K. A., Eachus, R. A., Ham, T. S., Kirby, J., Chang, M. C. Y., Withers, S. T., Shiba, Y., Sarpong, R., and Keasling, J. D. (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast, *Nature* 440(7086), 940–943. *https://doi:10.1038/nature04640*
4. Bourdeau, R. W., Lee-Gosselin, A., Lakshmanan, A., Farhadi, A., Kumar, S. R., Nety, S. P., and Shapiro, M. G. (2018) Acoustic reporter genes for noninvasive imaging of microorganisms in mammalian hosts, *Nature* 553(7686), 86–90. *https://doi:10.1038/nature25021*
5. Liu, W., and Stewart, C. N. (2015) Plant synthetic biology, *Trends in Plant Science* 20(5), 309–317. *https://doi:10.1016/j.tplants.2015.02.004*
6. Karig, D. K. (2017) Cell-free synthetic biology for environmental sensing and remediation, *Current Opinion in Biotechnology* 45, 69–75. *https://doi:10.1016/j.copbio.2017.01.010*
7. Clomburg, J. M., and Gonzalez, R. (2010) Biofuel production in *Escherichia coli*: the role of metabolic engineering and synthetic biology, *Applied Microbiology and Biotechnology* 86(2), 419–434. *https://doi:10.1007/s00253-010-2446-1*
8. Mutalik, V. K., Guimaraes, J. C., Cambray, G., Lam, C., Christoffersen, M. J., Mai, Q.-A., Tran, A. B., Paull, M., Keasling, J. D., Arkin, A. P., and Endy, D. (2013) Precise and reliable gene expression via standard transcription and translation initiation elements, *Nature Methods* 10(4), 354–360. *https://doi:10.1038/nmeth.2404*
9. Ruiz, R. C. H., Kiatwuthinon, P., Kahn, J. S., Roh, Y. H., and Luo, D. (2012) Cell-Free Protein Expression from DNA-Based Hydrogel (P-Gel) Droplets for Scale-Up Production, *Industrial Biotechnology* 8(6), 372–377. *https://doi:10.1089/ind.2012.0024*
10. Yue, S., Jie, J., Xie, L., Li, Y., Zhang, J., Lai, X., Xie, J., Guo, X., and Zhai, Y. (2020) Antimicrobial peptide CAMA-syn expressed in pulmonary epithelium by recombination adenovirus inhibited the growth of intracellular bacteria, *The Journal of Gene Medicine* 22(3). *https://doi:10.1002/jgm.3149*
11. Lagassé, H. A. D., Alexaki, A., Simhadri, V. L., Katagiri, N. H., Jankowski, W., Sauna, Z. E., and Kimchi-Sarfaty, C. (2017) Recent advances in (therapeutic protein) drug development. F1000Research, 6, 113. *https://doi:10.12688/f1000research.9970.1*
12. Lim, H. J., Park, Y. J., Jang, Y. J., Choi, J. E., Oh, J. Y., Park, J. H., Song, J. K., and Kim, D.-M. (2016) Cell-free synthesis of functional phospholipase A1 from Serratia sp., *Biotechnology for Biofuels* 9(1). *https://doi:10.1186/s13068-016-0563-5*
13. Merlin, M., Gecchele, E., Capaldi, S., Pezzotti, M., and Avesani, L. (2014) Comparative Evaluation of Recombinant Protein Production in Different Biofactories: The Green Perspective, *BioMed Research International* 2014, 1–14. *https://doi:10.1155/2014/136419*
14. Krinsky, N., Kaduri, M., Shainsky-Roitman, J., Goldfeder, M., Ivanir, E., Benhar, I., Shoham, Y., and Schroeder, A. (2016) A simple and rapid method for preparing a cell-free bacterial lysate for protein synthesis, *PLOS ONE* 11(10), e0165137. *https://doi:10.1371/journal.pone.0165137*
15. Kigawa, T., Yabuki, T., Matsuda, N., Matsuda, T., Nakajima, R., Tanaka, A., and Yokoyama, S. (2004) Preparation of *Escherichia coli* cell extract for highly productive cell-free protein expression, *Journal of Structural and Functional Genomics* 5(1/2), 63–68. *https://doi:10.1023/b:jsfg.0000029204.57846.7d*
16. Hino, M., Kataoka, M., Kajimoto, K., Yamamoto, T., Kido, J.-I., Shinohara, Y., and Baba, Y. (2008) Efficiency of cell-free protein synthesis based on a crude cell extract from *Escherichia coli*, wheat germ, and rabbit reticulocytes, *Journal of Biotechnology* 133(2), 183–189. *https://doi:10.1016/j.jbiotec.2007.08.008*
17. Kim, T.-W., Kim, D.-M., and Choi, C.-Y. (2006) Rapid production of milligram quantities of proteins in a batch cell-free protein synthesis system, *Journal of Biotechnology* 124(2), 373–380. *https://doi:10.1016/j.jbiotec.2005.12.030*
18. Caschera, F., and Noireaux, V. (2015) Preparation of amino acid mixtures for cell-free expression systems, *BioTechniques* 58(1). *https://doi:10.2144/000114249*

ABOUT STUDENT AUTHORS
The team of student authors were all undergraduates at the time they conducted the research. Some worked at Missouri Western State University (MWSU) whereas others worked at Davidson College (DC). They were funded by a pair of collaborative grants

that allowed them to visit each other's campuses during the summer of their research. Owen Koucky was a genomics major whose senior thesis focused on optimizing CFPS. Students at MWSU specialized in droplet production and droplet CFPS. Students at DC specialized in pooled CFPS. Students on both campuses conducted the DNA cloning.

PRESS SUMMARY

This synthetic biology study examines the reliability of protein production performed *in vitro*. Specifically, we compared the function of 20 bicistronic designs (BCDs) developed for use inside *E. coli* cells to their function in cell-free protein synthesis. We found that BCDs functioned similarly *in vitro* and *in vivo*. When tested within nanoliter scaled droplets produced by microfluidics, the BCDs also performed well, though GFP detection varied. In summary, BCDs can be used interchangeably for *in vivo* and *in vitro* protein production.

Survey of *Wolbachia* Frequency in Nashville, Tennessee Reveals Novel Infections

*Sangami Pugazenthi, Phoebe White, Aakash Basu, Anoop Chandrashekar, & J. Dylan Shropshire**

Department of Biological Sciences, Vanderbilt University, Nashville, TN 37235, USA

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Students: sangamithra.pugazenthi@vanderbilt.edu, phoebe.a.white@vanderbilt.edu, aakash.basu@vanderbilt.edu, anoop.chandrashekar@vanderbilt.edu
*Mentor: shropxp@gmail.com**

ABSTRACT
Wolbachia (Rickettsiales: Anaplasmataceae) are maternally transmitted intracellular bacteria that infect approximately half of all insect species. These bacteria commonly act as reproductive parasites or mutualists to enhance their transmission from mother to offspring, resulting in high prevalence among some species. Despite decades of research on *Wolbachia*'s global frequency, there are many arthropod families and geographic regions that have not been tested for *Wolbachia*. Here, arthropods were collected on the Vanderbilt University campus in Nashville, Tennessee, where *Wolbachia* frequency has not been previously studied. The dataset consists of 220 samples spanning 34 unique arthropod families collected on the Vanderbilt University campus. The majority of our samples were from the families Blattidae (Blattodea), Pulicidae (Siphonaptera), Dryinidae (Hymenoptera), Aphididae (Hemiptera), Paronellidae (Entomobryomorpha), Formicidae (Hymenoptera), Pseudococcidae (Hemiptera), Sphaeroceridae (Diptera), and Coccinellidae (Coleoptera). PCR-based techniques were used to assign infection states and, from these data, the first cases of *Wolbachia* in the Paronellidae springtails, Lithobiidae (Lithobiomorpha) centipedes, Lonchopteridae (Diptera) spear-winged flies, Sepsidae (Diptera) black scavenger flies, Cryptocercidae (Blattodea) wood roaches, and Lauxaniidae (Diptera) acalyptrate flies were identified. Within-family infection frequencies ranged from 17-100% when *Wolbachia* was observed; however, numerous families tested did not reveal evidence of infection. These results expand on the field's understanding of *Wolbachia*'s frequency in Nashville, Tennessee, and among arthropod families broadly, and is the first report of *Wolbachia* in centipedes.

KEYWORDS
Wolbachia; Infection Frequency; Endosymbiont; Tennessee; Centipede; Arthropod; Polymerase Chain Reaction; Nashville

INTRODUCTION

Wolbachia is a genus of obligate intracellular bacteria that commonly infect arthropods and nematodes.[1–4] They often reside in reproductive tissue cells, are vertically inherited from ova to offspring[5] and are occasionally transferred horizontally between arthropods.[6–9] *Wolbachia* frequently interact with their hosts as mutualists[10] and/or reproductive parasites[11] to encourage their proliferation through host populations.[12–17] *Wolbachia* can increase or decrease host longevity,[18–22] suppress pathogen replication,[23–27] provide essential nutrients to their host,[28, 29] and cause reproductive parasitism phenotypes such as cytoplasmic incompatibility, male-killing, feminization, and parthenogenesis.[11, 30–34]

Studies agree that *Wolbachia* are common among arthropod species.[1–4] However, estimates for the percentage of infected species range from 16.9%[1] to as high as 66%,[2] with other estimates reporting 40%[3] and 52%.[4] The variance in these estimates can be attributed to differences in testing methodologies and data sets used in these analyses. Each analysis leverages PCR-based screens for bacterial symbionts, but each differ in how they handle species that have small or large sample sizes since high sample sizes are more likely to reveal infections. Intra-species infection frequencies can be below 10% to as high as 100%,[3, 35] and these frequencies can vary based on geography.[36–39]

Despite decades of research aimed at elucidating *Wolbachia's* frequency among arthropods, estimates remain variable partly due to the lack of sampling of some arthropod families and in various geographic regions. Here, we aim to characterize *Wolbachia* infection frequency in arthropod families collected in Nashville, Tennessee, which, to our knowledge, has not been studied in the context of *Wolbachia* frequency. We collect arthropods, extract DNA, and use PCR-based techniques to describe infection states. We report that 73% of the families we collected are infected, identify *Wolbachia* in six families previously not reported with an infection, and describe infection frequencies ranging from 17-100% within infected families. These results expand our knowledge of the infection frequency of *Wolbachia* in a variety of arthropods in Nashville, Tennessee.

METHODS AND PROCEDURES

Arthropod collection and identification.

As part of an Introduction to Biology Laboratory course at Vanderbilt University, arthropods were collected from January to March 2018 on the Vanderbilt University campus in Nashville, Tennessee **(Fig. 1A)**. Samples were collected inside buildings, outside buildings on the campus grounds, and in the Vanderbilt University greenhouse **(Fig. 1B).** Each sample represents a single arthropod. A variety of collection methods were used, including Berlese funnels, food and chemical attractants, pitfall traps, color traps, sticky traps, and active collection. Arthropods were removed from traps within 24 h and were individually frozen at -20°C in sterile 1.5 mL Eppendorf tubes (Eppendorf, Hamburg, Germany), 15 mL conical tubes, or 50 mL conical tubes. The family of each arthropod was determined using dichotomous keys and pictorial guides.[40–42] Sub-family level identification was not conducted due to time restrictions of the class, and all specimens were destroyed during downstream processing.

Determining Arthropod Infection Status.

DNA was extracted from all arthropod samples using the Gentra Puregene Tissue Kit (QIAGEN, Hilden, Germany) slightly modified from the manufacturer's protocol. For larger samples, the posterior end of the arthropod was dissected for DNA extraction since *Wolbachia* are transmitted maternally and are likely to be present in these tissues. To determine infection state, PCR was conducted using WSpec-forward (5'-CAT ACC TAT TCG AAG GGA TAG-3') and WSpec-reverse (5'-AGC TTC GAG TGA AAC CAA TTC-3') primers targeting the *Wolbachia* 16S rRNA gene,[1, 43] using the following cycling conductions: 94℃ for 2 m, 30 cycles at 94℃ for 30 s, 49℃ for 45 s and 72℃ for 1 min, and a final extension at 72℃ for 10 m. Positive controls and negative controls were *Wolbachia*-infected and uninfected *Drosophila melanogaster* respectively from laboratory stocks generously donated by the Bordenstein lab at Vanderbilt University. PCR products were run on a 1% agarose gel and visualized under UV light after treatment with GelRed (Biotium, Fremont, CA). A sample was considered infected if it produced a 436 bp-long fragment as expected for the WSpec amplicon.

Analyses, and figure creation.

All analyses were conducted and graphs generated in GraphPad Prism 8 (GraphPad Software, San Diego, CA), maps were generated in ArcGIS Online (Esri, Redlands, CA), and figure aesthetics were edited in Affinity Designer 1.7 (Serif, Nottingham, United Kingdom).

RESULTS

Arthropods (n=220) were collected on the Vanderbilt University campus in Nashville, Tennessee (Fig. 1A). Samples were collected in three clearly different environments, either inside buildings, not including the Vanderbilt University greenhouse (n=21), outside buildings (n=84), or in the Vanderbilt University greenhouse (n=115), and most families collected were only found in one location type. The Psychodidae (Diptera) and Sphecidae (Hymenoptera) were only collected inside campus buildings; the Aphididae, Armadillidiidae (Isopoda), Cryptocercidae, Paronellidae, Pholcidae (Araneae), Pseudococcidae, and Thripidae (Thysanoptera) were only found in the greenhouse. The Acrididae (Orthoptera), Anthomyzidae (Diptera), Ceratopogonidae (Diptera), Dryinidae, Latridiidae (Coleoptera), Lauxaniidae, Lithobiidae, Lonchopteridae, Pulicidae (Siphonaptera), Salticidae (Araneae), Sarcophagidae (Diptera), Sciaridae (Diptera), Sepsidae, Sphaeroceridae (Diptera), Tetranychidae (Trombidiformes), Tipulidae (Diptera), and Vespidae (Hymenoptera) were only found on campus grounds (Fig. 1B). Additionally, Blattidae specimens were found in the greenhouse and non-greenhouse buildings, and Coccinellidae, Drosophilidae (Diptera), Formicidae, Muscidae (Diptera), Pentatomidae (Hemiptera), and Theridiidae (Araneae) specimens were found in both indoor and outdoor locations **(Fig. 1B).** The Phoridae (Diptera) were the only family found both in the greenhouse and on campus grounds **(Fig. 1B).**

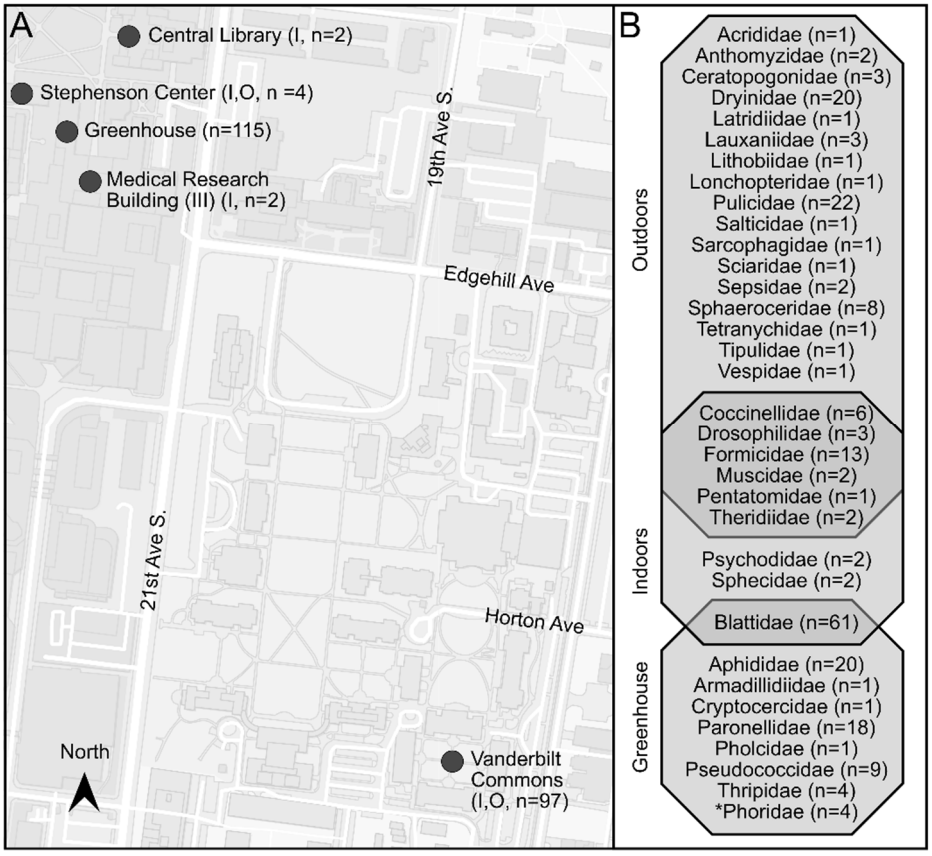

Figure 1. Samples representing 34 arthropod families were collected outdoors, indoors, and in a greenhouse on the Vanderbilt University campus in Nashville, TN. (A) Dark grey dots represent sampling locations. "I" indicates that samples were collected from inside buildings (excluding the greenhouse). "O" indicates that samples were collected outside and around campus buildings. The sample size is shown beside each sampling location. (B) Venn diagram shows arthropod families found in each sampling location. 17 families were only sampled from outdoor locations, two families only from indoor locations, and seven families only from the greenhouse. Some families are found both outside and inside, and some are found both inside and in the greenhouse. Only the Phoridae (denoted with an *) was found in both the greenhouse and outdoors. The number of samples collected for each family are shown to the right of the family name.

To characterize *Wolbachia* infection states, DNA was extracted from samples and PCR was used to amplify for a segment of the *Wolbachia* 16S rRNA gene using the WSpec primer set.[36, 43] We identified *Wolbachia* 16S rRNA fragments in 49% of specimens and in 73% of families. *Wolbachia* was not detected in the families Latridiidae (n=1), Pentatomidae (n=1), Salticidae (n=1), Tipulidae (n=1), Vespidae (n=1), Anthomyzidae (n=2), Sphecidae (n=2), Theridiidae (n=2), and Thripidae (n=4) **(Fig. 2**). However, aside from the Thripidae, all of the uninfected families in our study had two or fewer samples each, making it impossible to conclude whether these infection states can be generalized to the family as a whole. Additionally, *Wolbachia* was found in the families Acrididae (n=1, 100% infected), Armadillidiidae (n=1, 100%), Cryptocercidae (n=1, 100%), Lithobiidae (n=1, 100%), Lonchopteridae (n=1, 100%), Pholcidae (n=1, 100%), Sarcophagidae (n=1, 100%), Sciaridae (n=1, 100%), Tetranychidae (n=1, 100%), Muscidae (n=2, 50%), Psychodidae (n=2, 50%), Sepsidae (n=2, 50%), Ceratopogonidae (n=3, 33%), Drosophilidae (n=3, 67%), Lauxaniidae (n=3, 100%), Phoridae (n=4, 75%), Coccinellidae (n=6, 17%), Sphaeroceridae (n=8, 50%), Pseudococcidae (n=9, 100%), Formicidae (n=13, 31%), Paronellidae (n=18, 67%), Aphididae (n=20, 20%), Dryinidae (n=20, 70%), Pulicidae (n=22, 32%), Blattidae (n=61, 52%) **(Fig. 2)**. Among infected families, the infection frequency was as low as 17% in the Coccinellidae to as high as 100% in the families Acrididae, Armadillidiidae, Cryptocercidae, Lithobiidae, Lonchopteridae, Pholcidae, Sarcophagidae, Sciaridae, Tetranychidae, Lauxaniidae, and Pseudococcidae **(Fig. 2)**. Notably, we do not claim that *Wolbachia* has reached fixation in any of these families since all families with 100% infection frequency had low sample sizes.

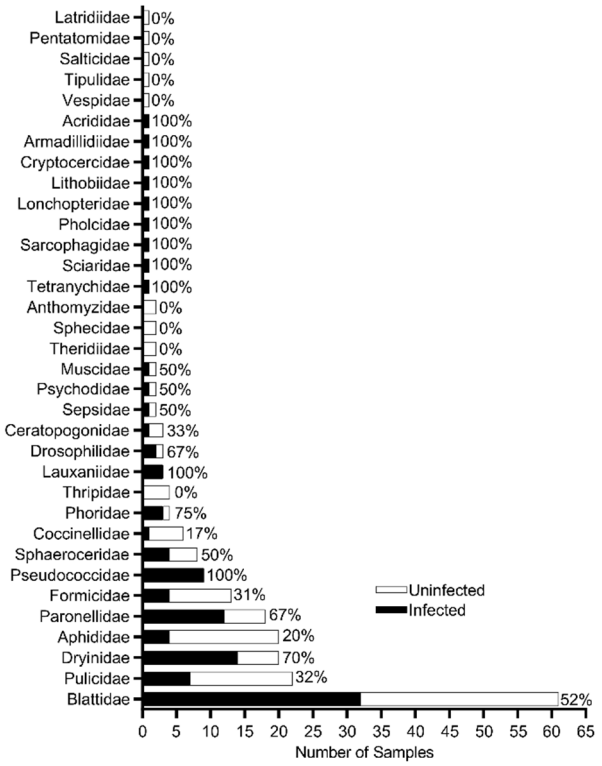

Figure 2. *Wolbachia* infection frequency varies between arthropod families. Of the 34 families collected, 25 were infected with *Wolbachia*. All of the samples were infected in ten families and all were uninfected in nine. The remaining families had infection frequencies between 17-67%. White bars represent uninfected samples and black bars represent infected samples. The percentage to the right of each bar represent the percent of infected samples per family.

DISCUSSION

We report *Wolbachia* infection states for 220 arthropod samples spanning 34 families, of which 25 are infected. To our knowledge, six of these infected families did not have prior reports of infection in the literature **(Table 1)**: Paronellidae elongate springtails, Lithobiidae stone centipedes, Lonchopteridae spear-winged flies, Sepsidae black scavenger flies, Crytptocercidae wood roaches, and Lauxaniidae acalyptrate flies. We found the infection frequency for these families to be 78% in the Paronellidae (n=18), 50% in the Sepsidae, and 100% in the Lithobiidae (n=1), Lonchopteridae (n=1), Cryptocercidae (n=1), and Lauxaniidae (n=4). Conclusions about infection frequencies can only be drawn from the Paronellidae, which had a relatively robust sample size. While not reported in the Paronellidae, *Wolbachia* has been reported in seven other springtail families spanning four orders including the order Entomobryomorpha, which contains the Paronellidae.[44–46] These results suggest that *Wolbachia* might be more common among this subclass than it would seem from literature.

The infection in Lithobiidae centipedes is of particular interest since, to our knowledge, *Wolbachia* has not been previously reported in centipedes. In fact, two prior surveys have proposed negative infection states for centipedes in the family Lithobiidae[36] and in the order Scolopendromorpha.[47] However, it remains possible that our positive infection state is the result of a false-positive infection. For instance, the carnivorous lifestyle of centipedes may increase the rate of false-positives since they may feed on *Wolbachia*-infected insects whose DNA would then contaminate the sample. Future studies that collect more samples and dissect specific tissue for *Wolbachia*-infection assays will help confirm the frequency of infection among this arthropod order. Additionally, while *Wolbachia* are common among Diptera and Blattodea,[12, 36, 48–50] we report the first cases of infection in the Lonchopteridae, Sepsidae, Lauzaniidae, and Cryptocercidae, which have been mostly overlooked. These results validate the premise that additional sampling and *Wolbachia* infection testing is necessary to identify *Wolbachia* in arthropod groups that are under sampled.

Finally, while there are reports of infection in the Pentatomidae,[51] Salticidae,[52] Theridiidae,[53, 54] Thripidae,[55–57] Tipulidae,[58] and Vespidae[59] in the literature **(Table 1)**, we did not find evidence of infection in these families in Nashville. However, we are cautious to make firm conclusions on these data due to low sample sizes and numerous alternative explanations for negative results. For example, PCR-based techniques can miss low-density *Wolbachia* infections if they are below a detection threshold,[60] failures in DNA extraction and/or PCR can result in false-negatives due to insufficient or low quality DNA, and since the WSpec primers used here were designed for Supergroup A and B *Wolbachia* it is plausible that our techniques would not detect highly divergent *Wolbachia* strains.[36, 43] Further work is necessary to confirm that *Wolbachia* do not reside in these families in Nashville, but if these results hold

to larger sampling it may suggest differences in *Wolbachia*'s frequency in Nashville relative to other regions that have been sampled. However, since we only identified samples to family, it is possible that our samples belong to different species than have been reported in other studies.

Family	Infected?	Literature reports of infection?	Reference
Acrididae	Yes	Yes	[61]
Aphididae	Yes	Yes	[62–64]
Armadillidiidae	Yes	Yes	[65]
Blattidae	Yes	Yes	[48, 49]
Ceratopogonidae	Yes	Yes	[66, 67]
Coccinellidae	Yes	Yes	[68]
Drosophilidae	Yes	Yes	[12, 69, 70]
Dryinidae	Yes	Yes	[71]
Formicidae	Yes	Yes	[72–74]
Muscidae	Yes	Yes	[75]
Pholcidae	Yes	Yes	[54]
Phoridae	Yes	Yes	[76]
Pseudococcidae	Yes	Yes	[77]
Psychodidae	Yes	Yes	[78–80]
Pulicidae	Yes	Yes	[81]
Sarcophagidae	Yes	Yes	[82]
Sciaridae	Yes	Yes	[83]
Sphaeroceridae	Yes	Yes	[59]
Tetranychidae	Yes	Yes	[84]
Lithobiidae	Yes	No	[36]
Sepsidae	Yes	No	[85]
Cryptocercidae	Yes	---	N/A
Lauxaniidae	Yes	---	N/A
Lonchopteridae	Yes	---	N/A
Paronellidae	Yes	---	N/A
Pentatomidae	No	Yes	[51]
Salticidae	No	Yes	[52]
Theridiidae	No	Yes	[53, 54]
Thripidae	No	Yes	[55–57]
Tipulidae	No	Yes	[58]
Vespidae	No	Yes	[59]
Sphecidae	No	No	[36]
Anthomyzidae	No	---	N/A
Latridiidae	No	---	N/A

Table 1. Family level *Wolbachia* infection status compared to the literature. Bold family names represent families for which the infection status in the literature disagrees with those reported in this study. Bold and underlined families had not been previously screened for *Wolbachia* infection.

In summary, we describe *Wolbachia*'s frequency among arthropods in Nashville, Tennessee and report the first instance of *Wolbachia* in several arthropod families, including in the Lithobiidae centipedes. Additionally, we provide data for infection frequency within numerous other families, often overlooked by the current literature. This research will inform studies aimed at understanding *Wolbachia*'s global spread and distribution, by adding Nashville, Tennessee, to the *Wolbachia* pandemic map.

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Author contributions

JDS designed research; SP, PW, AB, AC, and JDS performed research and analyzed data; SP, PW, AB, and JDS wrote the paper.

REFERENCES

1. Werren JH, Windsor D, Guo LR. (1995) Distribution of *Wolbachia* among neotropical arthropods. *Proc R Soc Lond B Biol Sci.*; 262(1364):197-204. *https://doi:10.1098/rspb.1995.0196*

2. Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH. (2008) How many species are infected with *Wolbachia*? – a statistical analysis of current data. *Fems Microbiol Lett.*; 281(2):215-220. *https://doi:10.1111/j.1574-6968.2008.01110.x*

3. Zug R, Hammerstein P. (2012) Still a Host of Hosts for *Wolbachia*: Analysis of Recent Data Suggests That 40% of Terrestrial Arthropod Species Are Infected. *PLOS One.*; 7(6). *https://doi:10.1371/journal.pone.0038544*

4. Weinert LA, Araujo-Jnr EV, Ahmed MZ, Welch JJ. (2015) The incidence of bacterial endosymbionts in terrestrial arthropods. *Proc Biol Sci.*; 282(1807):20150249. *https://doi:10.1098/rspb.2015.0249*

5. Serbus LR, Casper-Lindley C, Landmann F, Sullivan W. (2008) The genetics and cell biology of *Wolbachia*-host interactions. *Annu Rev Genet.*; 42:683-707. *https://doi:10.1146/annurev.genet.41.110306.130354*

6. Boyle L, O'Neill SL, Robertson HM, Karr TL. (1993) Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila. Science.*; 260(5115):1796-1799. *https://doi:10.1126/science.8511587*

7. Gerth M, Röthe J, Bleidorn C. (2013) Tracing horizontal *Wolbachia* movements among bees (Anthophila): a combined approach using multilocus sequence typing data and host phylogeny. *Mol Ecol.*; 22(24):6149-6162. *https://doi:10.1111/mec.12549*

8. Wang N, Jia S, Xu H, Liu Y, Huang D. (2016) Multiple Horizontal Transfers of Bacteriophage WO and Host *Wolbachia* in Fig Wasps in a Closed Community. *Front Microbiol.*; 7. *https://doi:10.3389/fmicb.2016.00136*

9. Tolley SJA, Nonacs P, Sapountzis P. (2019) *Wolbachia* Horizontal Transmission Events in Ants: What Do We Know and What Can We Learn? *Front Microbiol.*; 10. *https://doi:10.3389/fmicb.2019.00296*

10. Zug R, Hammerstein P. (2015) Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. *Biol Rev Camb Philos Soc.*; 90(1):89-111. *https://doi:10.1111/brv.12098*

11. Werren JH, Baldo L, Clark ME. (2008) *Wolbachia*: master manipulators of invertebrate biology. *Nat Rev Microbiol.*; 6(10):741-751. *https://doi:10.1038/nrmicro1969*

12. Clark ME, Anderson CL, Cande J, Karr TL. (2005) Widespread Prevalence of *Wolbachia* in Laboratory Stocks and the Implications for *Drosophila* Research. *Genetics.*;170(4):1667-1675. *https://doi:10.1534/genetics.104.038901*

13. Hancock PA, Sinkins SP, Godfray HCJ. (2011) Population dynamic models of the spread of *Wolbachia. Am Nat.*;177(3):323-333. *https://doi:10.1086/658121*

14. Cordaux R, Pichon S, Hatira HBA, et al. (2012) Widespread *Wolbachia* infection in terrestrial isopods and other crustaceans. *ZooKeys.*;(176):123-131. *https://doi:10.3897/zookeys.176.2284*

15. Jiggins FM. (2017) The spread of *Wolbachia* through mosquito populations. *PLOS Biol.*;15(6):e2002780. *https://doi:10.1371/journal.pbio.2002780*

16. Bakovic V, Schebeck M, Telschow A, Stauffer C, Schuler H. (2018) Spatial spread of *Wolbachia* in *Rhagoletis cerasi* populations. *Biol Lett.*;14(5). *https://doi:10.1098/rsbl.2018.0161*

17. Turelli M, Cooper BS, Richardson KM, et al. (2018) Rapid Global Spread of *w*Ri-like *Wolbachia* across Multiple *Drosophila. Curr Biol CB.*;28(6):963-971.e8. *https://doi:10.1016/j.cub.2018.02.015*

18. Min K-T, Benzer S. (1997) *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proc Natl Acad Sci U S A.*;94(20):10792-10796.

19. Fry AJ, Palmer MR, Rand DM. (2004) Variable fitness effects of *Wolbachia* infection in *Drosophila melanogaster. Heredity.*;93(4):379-389. *https://doi:10.1038/sj.hdy.6800514*

20. Alexandrov ID, Alexandrova MV, Goryacheva II, Rochina NV, Shaikevich EV, Zakharov IA. (2007) Removing endosymbiotic *Wolbachia* specifically decreases lifespan of females and competitiveness in a laboratory strain of *Drosophila melanogaster. Russ J Genet.*;43(10):1147-1152. *https://doi:10.1134/S1022795407100080*

21. Vaĭsman NI, Ilinskiĭ II, Golubovskiĭ MD. (2009) Population genetic analysis of *Drosophila melanogaster* longevity: similar effects of endosymbiont *Wolbachia* and tumor suppressor lgl under conditions of temperature stress. *Zh Obshch Biol.*;70(5):438-447.

22. Maistrenko OM, Serga SV, Vaiserman AM, Kozeretska IA. (2016) Longevity-modulating effects of symbiosis: insights from *Drosophila–Wolbachia* interaction. *Biogerontology.*;17(5):785-803. *https://doi:10.1007/s10522-016-9653-9*

23. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, et al. (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and Plasmodium. *Cell.*;139(7):1268-1278. *https://doi:10.1016/j.cell.2009.11.042*

24. Hoffmann AA, Montgomery BL, Popovici J, et al. (2011) Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature.*;476(7361):454-457. *https://doi:10.1038/nature10356*

25. Hughes GL, Koga R, Xue P, Fukatsu T, Rasgon JL. (2011) *Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. *PLoS Pathog.*;7(5):e1002043. *https://doi:10.1371/journal.ppat.1002043*

26. Caragata EP, Dutra HLC, Moreira LA. (2016) Exploiting Intimate Relationships: Controlling Mosquito-Transmitted Disease with *Wolbachia. Trends Parasitol.*;32(3):207-218. *https://doi:10.1016/j.pt.2015.10.011*

27. Ant TH, Herd CS, Geoghegan V, Hoffmann AA, Sinkins SP. (2018) The *Wolbachia* strain *w*Au provides highly efficient virus transmission blocking in *Aedes aegypti. PLoS Pathog.*;14(1):e1006815. *https://doi:10.1371/journal.ppat.1006815*

28. Hosokawa T, Koga R, Kikuchi Y, Meng X-Y, Fukatsu T. (2010) *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc Natl Acad Sci.*;107(2):769-774. *https://doi:10.1073/pnas.0911476107*

29. Moriyama M, Nikoh N, Hosokawa T, Fukatsu T. (2015) Riboflavin Provisioning Underlies *Wolbachia*'s Fitness Contribution to Its Insect Host. *mBio*.;6(6):e01732-01715. *https://doi:10.1128/mBio.01732-15*

30. Yen JH, Barr AR. (1973) The etiological agent of cytoplasmic incompatibility in *Culex pipiens*. *J Invertebr Pathol*.;22(2):242-250. doi:10.1016/0022-2011(73)90141-9

31. Rousset F, Bouchon D, Pintureau B, Juchault P, Solignac M. (1992) *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proc Biol Sci*.;250(1328):91-98. *https://doi:10.1098/rspb.1992.0135*

32. Stouthamer R, Breeuwert JA, Luck RF, Werren JH. (1993) Molecular identification of microorganisms associated with parthenogenesis. *Nature*. ;361(6407):66-68. *https://doi:10.1038/361066a0*

33. Hurst GDD, Jiggins FM, Schulenburg JHG von der, et al. (1999) Male-killing *Wolbachia* in two species of insect. *Proc R Soc B Biol Sci*.;266(1420):735. *https://doi:10.1098/rspb.1999.0698*

34. LePage D, Bordenstein SR. (2013) *Wolbachia*: Can we save lives with a great pandemic? *Trends Parasitol*.;29(8):385-393. *https://doi:10.1016/j.pt.2013.06.003*

35. Sazama EJ, Ouellette SP, Wesner JS. (2019) Bacterial Endosymbionts Are Common Among, but not Necessarily Within, Insect Species. *Environ Entomol*.;48(1):127-133. *https://doi:10.1093/ee/nvy188*

36. Werren JH, Windsor DM. (2000) *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? *Proc R Soc B Biol Sci*.;267(1450):1277-1285.

37. Duplouy A, Couchoux C, Hanski I, van Nouhuys S. (2015) *Wolbachia* Infection in a Natural Parasitoid Wasp Population. Bourtzis K, ed. *PLOS ONE*.;10(8):e0134843. *https://doi:10.1371/journal.pone.0134843*

38. Schuler H, Köppler K, Daxböck-Horvath S, et al. (2016) The hitchhiker's guide to Europe: the infection dynamics of an ongoing *Wolbachia* invasion and mitochondrial selective sweep in *Rhagoletis cerasi*. *Mol Ecol*.;25(7):1595-1609. *https://doi:10.1111/mec.13571*

39. Schuler H, Egan SP, Hood GR, Busbee RW, Driscoe AL, Ott JR. (2018) Diversity and distribution of *Wolbachia* in relation to geography, host plant affiliation and life cycle of a heterogonic gall wasp. *BMC Evol Biol*.;18(1):37. *https://doi:10.1186/s12862-018-1151-x*

40. American Museum of Natural History. Biodiversity Counts. https://www.amnh.org/learn/biodiversity_counts/ident_help/Text_Keys/text_keys_index.htm (Accessed March 8, 2019)

41. BugGuide.Net. https://bugguide.net/node/view/15740 (Accessed March 8, 2019)

42. Zurqui All-Diptera Biodiversity Inventory. Zurqui All-Diptera Biodiversity Inventory. http://phorid.net/zadbi/ (Accessed March 8, 2019)

43. Sakamoto JM, Feinstein J, Rasgon JL. (2006) *Wolbachia* Infections in the *Cimicidae*: Museum Specimens as an Untapped Resource for Endosymbiont Surveys. *Appl Environ Microbiol*.;72(5):3161-3167. *https://doi:10.1128/AEM.72.5.3161-3167.2006*

44. Vandekerckhove TTM, Watteyne S, Willems A, Swings JG, Mertens J, Gillis M. (1999) Phylogenetic analysis of the 16S rDNA of the cytoplasmic bacterium *Wolbachia* from the novel host *Folsomia candida* (Hexapoda, Collembola) and its implications for wolbachial taxonomy. *FEMS Microbiol Lett*.;180(2):279-286. *https://doi:10.1111/j.1574-6968.1999.tb08807.x*

45. Ma Y, Chen W, Li Z, Zhang F, Gao Y, Luan Y. (2017) Revisiting the phylogeny of *Wolbachia* in Collembola. *Ecol Evol*.;7(7):2009-2017. *https://doi:10.1002/ece3.2738*

46. Czarnetzki AB, Tebbe CC. (2004) Detection and phylogenetic analysis of *Wolbachia* in Collembola. *Environ Microbiol*.;6(1):35-44. *https://doi:10.1046/j.1462-2920.2003.00537.x*

47. Simões PM. (2012) Diversity and dynamics of *Wolbachia*-host associations in arthropods from the Society archipelago, French Polynesia. *Doctoral dissertation. France: University of Lyon*.

48. Vaishampayan PA, Dhotre DP, Gupta RP, et al. (2007) Molecular evidence and phylogenetic affiliations of *Wolbachia* in cockroaches. *Mol Phylogenet Evol*.;44(3):1346-1351. *https://doi:10.1016/j.ympev.2007.01.003*

49. Gibson CM, Hunter MS. (2009) Inherited Fungal and Bacterial Endosymbionts of a Parasitic Wasp and Its Cockroach Host. *Microb Ecol*.;57(3):542-549. *https://doi:10.1007/s00248-008-9436-1*

50. Prezotto LF, Perondini ALP, Hernández-Ortiz V, Marino CL, Selivon D. (2017) *Wolbachia* strains in cryptic species of the *Anastrepha fraterculus* complex (Diptera, Tephritidae) along the Neotropical Region. *Syst Appl Microbiol*.;40(1):59-67. *https://doi:10.1016/j.syapm.2016.11.002*

51. Kikuchi Y, Fukatsu T. (2003) Diversity of *Wolbachia* Endosymbionts in Heteropteran Bugs. *Appl Environ Microbiol*.;69(10):6082-6090. *https://doi:10.1128/AEM.69.10.6082-6090.2003*

52. Zhang L, Zhang G, Yun Y, Peng Y. (2017) Bacterial community of a spider, *Marpiss magister* (Salticidae). *3 Biotech*.;7(6):371. *https://doi:10.1007/s13205-017-0994-0*

53. Rowley SM, Raven RJ, McGraw EA. (2004) *Wolbachia pipientis* in Australian Spiders. *ResearchGate*.;49(3):208-214. doi:10.1007/s00284-004-4346-z

54. Goodacre SL, Martin OY, Thomas CFG, Hewitt GM. (2006) *Wolbachia* and other endosymbiont infections in spiders. *Mol Ecol*.;15(2):517-527. *https://doi:10.1111/j.1365-294X.2005.02802.x*

55. Kumm S, Moritz G. (2008) First detection of *Wolbachia* in arrhenotokous populations of thrips species (Thysanoptera: Thripidae and Phlaeothripidae) and its role in reproduction. *Environ Entomol*.;37(6):1422-1428. https://doi:10.1603/0046-225x-37.6.1422

56. Rugman-Jones PF, Seybold SJ, Graves AD, Stouthamer R. (2015) Phylogeography of the Walnut Twig Beetle, *Pityophthorus juglandis*, the Vector of Thousand Cankers Disease in North American Walnut Trees. *PLOS ONE*.;10(2):e0118264. *https://doi:10.1371/journal.pone.0118264*

57. Saurav GK, Daimei G, Rana VS, Popli S, Rajagopal R. (2016) Detection and Localization of *Wolbachia* in *Thrips palmi* Karny (Thysanoptera: Thripidae). *Indian J Microbiol*.;56(2):167-171. *https://doi:10.1007/s12088-016-0567-7*

58. de Oliveira CD, Gonçalves DS, Baton LA, Shimabukuro PHF, Carvalho FD, Moreira LA. (2015) Broader prevalence of *Wolbachia* in insects including potential human disease vectors. *Bull Entomol Res*.;105(3):305-315. doi:10.1017/S0007485315000085

59. Stahlhut JK, Desjardins CA, Clark ME, et al. (2010) The mushroom habitat as an ecological arena for global exchange of *Wolbachia*. *Mol Ecol*.;19(9):1940-1952. *https://doi:10.1111/j.1365-294X.2010.04572.x*

60. Wolfgang A, Markus R, Dimitrios A, Christian S. Evidence for low-titre infections in insect symbiosis: *Wolbachia* in the bark beetle *Pityogenes chalcographus* (Coleoptera, Scolytinae). *Environ Microbiol*. 2009;11(8):1923-1933. *https://doi:10.1111/j.1462-2920.2009.01914.x*

61. Zabal-Aguirre M, Arroyo F, Bella JL. (2010) Distribution of *Wolbachia* infection in *Chorthippus parallelus* populations within and beyond a Pyrenean hybrid zone. *Heredity*.;104(2):174-184. *https://doi:10.1038/hdy.2009.106*

62. De Clerck C, Tsuchida T, Massart S, Lepoivre P, Francis F, Jijakli MH. (2014) Combination of Genomic and Proteomic Approaches to Characterize the Symbiotic Population of the Banana Aphid (Hemiptera: Aphididae). *Environ Entomol*.;43(1):29-36. *https://doi:10.1603/EN13107*

63. Wang Z, Su X-M, Wen J, Jiang L-Y, Qiao G-X. (2014) Widespread infection and diverse infection patterns of *Wolbachia* in Chinese aphids. *Insect Sci*.;21(3):313-325. *https://doi:10.1111/1744-7917.12102*

64. Moreira M, Aguiar AMF, Bourtzis K, Latorre A, Khadem M. (2019) *Wolbachia* (Alphaproteobacteria: Rickettsiales) Infections in Isolated Aphid Populations from Oceanic Islands of the Azores Archipelago: Revisiting the Supergroups M and N. *Environ Entomol*.;48(2):326-334. *https://doi:10.1093/ee/nvy189*

65. Cordaux R, Michel-Salzat A, Frelon-Raimond M, Rigaud T, Bouchon D. (2004) Evidence for a new feminizing *Wolbachia* strain in the isopod *Armadillidium vulgare*: evolutionary implications. *Heredity*.;93(1):78-84. *https://doi:10.1038/sj.hdy.6800482*

66. Mee PT, Weeks AR, Walker PJ, Hoffmann AA, Duchemin J-B. (2015) Detection of Low-Level *Cardinium* and *Wolbachia* Infections in *Culicoides*. *Appl Environ Microbiol*.;81(18):6177-6188. *https://doi:10.1128/AEM.01239-15*

67. Pagès N, Muñoz-Muñoz F, Verdún M, Pujol N, Talavera S. (2017) First detection of *Wolbachia*-infected *Culicoides* (Diptera: Ceratopogonidae) in Europe: *Wolbachia* and *Cardinium* infection across *Culicoides* communities revealed in Spain. *Parasit Vectors*.;10(1):582. *https://doi:10.1186/s13071-017-2486-9*

68. Sokolova MI, Zinkevich NS, Zakharov IA. (2002) Bacteria in ovarioles of females from maleless families of ladybird beetles *Adalia bipunctata* L. (Coleoptera: Coccinellidae) naturally infected with *Rickettsia*, *Wolbachia*, and *Spiroplasma*. *J Invertebr Pathol*.;79(2):72-79. *https://doi:10.1016/s0022-2011(02)00012-5*

69. Hamm CA, Begun DJ, Vo A, et al. (2014) *Wolbachia* do not live by reproductive manipulation alone: infection polymorphism in *Drosophila suzukii* and *D. subpulchrella*. *Mol Ecol*.;23(19):4871-4885. *https://doi:10.1111/mec.12901*

70. Bykov RA, Yudina MA, Gruntenko NE, et al. (2019) Prevalence and genetic diversity of *Wolbachia* endosymbiont and mtDNA in Palearctic populations of *Drosophila melanogaster*. *BMC Evol Biol*.;19(S1):48. *https://doi:10.1186/s12862-019-1372-9*

71. Espinosa MS, Virla EG, Cuozzo S. (2017) *Wolbachia* Infections Responsible for Thelytoky in Dryinid Wasps. The Case of *Gonatopus bonaerensis* Virla (Hymenoptera: Dryinidae). *Neotrop Entomol*.;46(4):409-413. *https://doi:10.1007/s13744-016-0475-x*

72. Reuter M, Keller L. (2003) High levels of multiple *Wolbachia* infection and recombination in the ant *Formica exsecta*. *Mol Biol Evol*.;20(5):748-753. *https://doi:10.1093/molbev/msg082*

73. Ramalho MO, Bueno OC, Moreau CS. (2017) Microbial composition of spiny ants (Hymenoptera: Formicidae: Polyrhachis) across their geographic range. *BMC Evol Biol*.;17(1):96. *https://doi:10.1186/s12862-017-0945-8*

74. Ramalho MO, Vieira AS, Pereira MC, Moreau CS, Bueno OC. (2018) Transovarian Transmission of *Blochmannia* and *Wolbachia* Endosymbionts in the Neotropical Weaver Ant *Camponotus textor* (Hymenoptera, Formicidae). *Curr Microbiol*.;75(7):866-873. *https://doi:10.1007/s00284-018-1459-3*

75. Zhang B, McGraw E, Floate KD, James P, Jorgensen W, Rothwell J. (2009) *Wolbachia* infection in Australasian and North American populations of *Haematobia irritans* (Diptera: Muscidae). *Vet Parasitol*.;162(3-4):350-353. *https://doi:10.1016/j.vetpar.2009.03.012*

76. Dedeine F, Ahrens M, Calcaterra L, Shoemaker DD. (2005) Social parasitism in fire ants (Solenopsis spp.): a potential mechanism for interspecies transfer of *Wolbachia*. *Mol Ecol*.;14(5):1543-1548. doi:10.1111/j.1365-294X.2005.02499.x

77. Tang Y, Wang C, Lu YY. (2013) First discovery of *Wolbachia* infection of cotton mealybug *Phenacoccus solenopsis* Tinsley. *J Environ Entomol*. 2013;1.

78. Cui L, Chang SH, Strickman D, Rowton E. (1999) Frequency of *Wolbachia* infection in laboratory and field sand fly (Diptera: Psychodidae) populations. *J Am Mosq Control Assoc*.;15(4):571-572.

79. Li K, Chen H, Jiang J, Li X, Xu J, Ma Y. (2016) Diversity of bacteriome associated with *Phlebotomus chinensis* (Diptera: Psychodidae) sand flies in two wild populations from China. *Sci Rep*.;6:36406. *https://doi:10.1038/srep36406*

80. Karimian F, Vatandoost H, Rassi Y, et al. (2018) *wsp*-based analysis of *Wolbachia* strains associated with *Phlebotomus papatasi* and *P. sergenti* (Diptera: Psychodidae) main cutaneous leishmaniasis vectors, introduction of a new subgroup *n*Serg. *Pathog Glob Health*.;112(3):152-160. *https://doi:10.1080/20477724.2018.1471438*

81. Zurita A, Gutiérrez SG, Cutillas C. (2016) Infection Rates of *Wolbachia* sp. and *Bartonella* sp. in Different Populations of Fleas. *Curr Microbiol*.;73(5):704-713. *https://doi:10.1007/s00284-016-1119-4*

82. Mingchay P, Sai-Ngam A, Phumee A, et al. (2014) *Wolbachia* supergroups A and B in natural populations of medically important filth flies (diptera: muscidae, calliphoridae, and sarcophagidae) in Thailand. *Southeast Asian J Trop Med Public Health*.;45(2):309-318.

83. Tao Y, Guo Y, Wang J, Li L, Yu Y, Chu D. (201) Detection and identification of *Wolbachia* in *Bradysia odoriphaga* (Diptera: Sciaridae) populations from Shandong Province, China. *Acta Entomol Sin*. 5;58(4):454-459.

84. Tsagkarakou A, Guillemaud T, Rousset F, Navajas M. (1996) Molecular identification of a *Wolbachia* endosymbiont in a *Tetranychus urticae* strain (Acari: Tetranychidae). *Insect Mol Biol*.;5(3):217-221. *https://doi:10.1111/j.1365-2583.1996.tb00057.x*

85. Floate KD, Kyei-Poku GK, Coghlin PC. (2006) Overview and relevance of *Wolbachia* bacteria in biocontrol research. *Biocontrol Sci Technol*.;16(8):767-788. *https://doi:10.1080/09583150600699606*

ABOUT STUDENT AUTHORS

Sangami Pugazenthi, Phoebe White, Aakash Basu, and Anoop Chandrashekar are undergraduate students at Vanderbilt University. Sangami and Aakash graduated in May 2020, while Phoebe and Anoop expect to graduate May 2021.

PRESS SUMMARY

Wolbachia are intracellular and maternally inherited bacteria that infect roughly half of all arthropod species. Since arthropods account for approximately 85% of all animals, *Wolbachia* is thought to be the world's most common animal-associated bacterial infection. However, estimates of *Wolbachia* infection frequency range from as low as 17% to as high as 66% of species. Additionally, strong arthropod family-biases and geographical-biases exist in the current literature. It is thus important to survey new species and different geographic regions to better understand *Wolbachia's* global frequency. Here, we report a dataset of 220 arthropod samples, spanning 34 families, that have been tested for *Wolbachia* using PCR-based techniques. These samples were collected by undergraduate students as part of an Introduction to Biology Laboratory at Vanderbilt University in Nashville, Tennessee. We confirm literature reports of infections in 25 arthropod families and novel infections in six, and put Nashville on the map of locations tested for *Wolbachia* frequency.

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