

**A peer-reviewed version of this preprint was published in PeerJ on 9 August 2016.**

[View the peer-reviewed version](https://peerj.com/articles/2316) (peerj.com/articles/2316), which is the preferred citable publication unless you specifically need to cite this preprint.

Shropshire JD, van Opstal EJ, Bordenstein SR. 2016. An optimized approach to germ-free rearing in the jewel wasp *Nasonia*. PeerJ 4:e2316  
<https://doi.org/10.7717/peerj.2316>

# An optimized approach to germ-free rearing in the jewel wasp *Nasonia*

J. Dylan Shropshire, Edward J. van Opstal, Seth R. Bordenstein

The initial development of the *Nasonia in vitro* germ-free rearing system in 2012 enabled investigations of *Nasonia*-microbiota interactions and real-time visualization of metamorphosis. However, the use of antibiotics, bleach, and fetal bovine serum in this *in vitro* rearing system introduced artifacts relative to conventional rearing of *Nasonia*. Here, we optimize the germ-free rearing procedure by using filter sterilization *in lieu* of antibiotics and by removing residual bleach and fetal bovine serum. Comparison of these methods reveals no influence on larval survival or growth, and a 52% improvement in adult production. Additionally, adult males produced in the new germ-free system are similar in size to conventionally reared males. Experimental implications of these changes are discussed.

1 **An Optimized Approach to Germ-free Rearing in the Jewel Wasp *Nasonia***

2 J. Dylan Shropshire<sup>1\*</sup>, Edward J. van Opstal<sup>1\*</sup>, and Seth R. Bordenstein<sup>1,2</sup>

3 <sup>1</sup>Vanderbilt University, Department of Biological Sciences, Nashville, TN, USA

4 <sup>2</sup>Vanderbilt University, Department of Pathology, Microbiology, and Immunology, Nashville,  
5 TN, USA

6 \*These authors contributed equally to this work

7

8 Corresponding Authors:

9 J. Dylan Shropshire

10 U7217 BSB / MRB III, 465 21<sup>st</sup> Ave South, Nashville, TN, 37232, USA

11 Email address: [dylan.shropshire@vanderbilt.edu](mailto:dylan.shropshire@vanderbilt.edu)

12 Seth R. Bordenstein

13 U7215 BSB / MRB III, 465 21<sup>st</sup> Ave South, Nashville, TN, 37232, USA

14 Email Address: [s.bordenstein@vanderbilt.edu](mailto:s.bordenstein@vanderbilt.edu)

15

16 **ABSTRACT**

17 The initial development of the *Nasonia in vitro* germ-free rearing system in 2012 enabled  
18 investigations of *Nasonia*-microbiota interactions and real-time visualization of metamorphosis.  
19 However, the use of antibiotics, bleach, and fetal bovine serum in this *in vitro* rearing system  
20 introduced artifacts relative to conventional rearing of *Nasonia*. Here, we optimize the germ-free  
21 rearing procedure by using filter sterilization *in lieu* of antibiotics and by removing residual  
22 bleach and fetal bovine serum. Comparison of these methods reveals no influence on larval  
23 survival or growth, and a 52% improvement in adult production. Additionally, adult males  
24 produced in the new germ-free system are similar in size to conventionally reared males.  
25 Experimental implications of these changes are discussed.

26

## 27 INTRODUCTION

28           The *Nasonia* genus (Ashmead & Smith, 1904) consists of four closely related interfertile  
29 parasitoid wasp species and has been a powerful model for the study of genetics (Davies &  
30 Tauber, 2015; Lynch, 2015; Raychoudhury et al., 2010), evolution (Bordenstein, O'Hara &  
31 Werren, 2001; Bordenstein & Werren, 2007; Brucker & Bordenstein, 2013; Clark et al., 2010),  
32 endosymbiosis (Bordenstein, O'Hara & Werren, 2001; Ferree et al., 2008), development (Rivers  
33 & Losinger, 2014; Verhulst et al., 2013; Zwier et al., 2012), behavior (Baeder & King, 2004;  
34 Beukeboom & van den Assem, 2001; Clark et al., 2010; Drapeau & Werren, 1999;  
35 Raychoudhury et al., 2010), pheromonal communication (Diao et al., 2016; Ruther & Hammerl,  
36 2014; Steiner, Hermann & Ruther, 2006), and other areas. The design and publication of an *in*  
37 *vitro* system for *Nasonia* in 2012 detached *Nasonia* from its fly host, allowed for real-time  
38 monitoring of development, and provided an avenue to study how microbes influence *Nasonia*  
39 biology (Brucker & Bordenstein, 2012a). These tools advanced the *Nasonia* system to explore  
40 how gut microbiota influence development and hybrid lethality (Brucker & Bordenstein, 2013).

41           *Nasonia* germ-free rearing involves two major components: (i) sterilizing *Nasonia*  
42 embryos and (ii) providing larvae with sterilized food in an *in vitro* system. Embryo sterilization  
43 is conducted by picking *Nasonia* embryos from pupal fly hosts (typically *Sarcophaga bullata*;  
44 Werren & Loehlin, 2009a) and then rinsing the embryos with bleach followed by sterile water  
45 (Brucker & Bordenstein, 2012a). Producing *Nasonia* Rearing Medium (NRM) involves the  
46 collection of hundreds of fly pupae, extraction of proteinaceous fluids from those pupae, addition  
47 of fetal bovine serum (FBS) and Schneider's *Drosophila* medium for additional nutrition, filter  
48 sterilization, and addition of antibiotics (Fig 1; Brucker & Bordenstein, 2012a). Sterilized

49 embryos are then placed on a transwell permeable membrane with filter-sterilized NRM  
50 underneath for feeding (Brucker & Bordenstein, 2012a).

51         This protocol yielded similarly sized *Nasonia* to those from *in vivo* rearing (Brucker &  
52 Bordenstein, 2012a). However, NRM production relies on introducing foreign and potentially  
53 harmful elements such as bleach, FBS, and antibiotics. Removal of each component carries its  
54 own rationale. For example, the bleach treatment was intended to kill surface bacteria on the  
55 puparium of host flies and remove particulates (Brucker & Bordenstein, 2012a). However,  
56 surface bacteria will be removed during filtration and residual bleach from the rinse may persist  
57 in the final NRM product as a toxic agent. Furthermore, FBS is added as a nutritional supplement  
58 to increase larval survival and development (Brucker & Bordenstein, 2012a), but *Nasonia* do not  
59 frequently encounter components of FBS including bovine-derived hormones such as  
60 testosterone, progesterone, insulin, and growth hormones (Honn, Singly & Chavin, 1975).  
61 Finally, antibiotics are a confounding variable and removing them will provide more flexibility  
62 to bacterial inoculations in the *in vitro* system.

63         This study removes these three major components of the original NRM and optimizes the  
64 procedure by eliminating extraneous steps and utilizing quicker approaches. These changes are  
65 validated by directly comparing germ-free *Nasonia* reared on either the original (NRMv1) or  
66 optimized (NRMv2) media for larval survival, larval growth, and adult production. The  
67 morphology of adults produced both *in vitro* and *in vivo* is then compared.

68

## 69 **MATERIALS AND METHODS**

70 *Nasonia rearing medium (NRMv1)*

71           *Sarcophaga bullata* pupae were produced as previously described (Werren & Loehlin,  
72 2009a). Approximately 150 ml of *S. bullata* pupae were transferred to a sterile beaker after close  
73 inspection to remove larvae, poor quality pupae, and debris. A solution of 10% Clorox bleach  
74 was then added to the beaker to cover the pupae. After five minutes, the bleach was drained from  
75 the beaker and the pupae were repeatedly rinsed with sterile millipore water until the scent of  
76 bleach was absent. Sterile millipore water was added in the beaker to approximately  $\frac{2}{3}$  the  
77 volume of pupae, covered, and placed in a 36°C water bath to soften the puparium. *S. bullata*  
78 pupae were homogenized using a household kitchen blender and filtered through a 100 µm cell  
79 strainer. The filtrate was centrifuged at 4°C (25,000xG) for 5 minutes to separate the sediment,  
80 protein, and lipid layers, and a 22 gauge needle was used to remove the protein layer. The protein  
81 layer was combined with 50 ml of Schneider's *Drosophila* medium 1 x and 20% FBS. The  
82 resulting product was passed through filters with gradually smaller pore sizes (11, 6, 2.5, 0.8, and  
83 0.45 µm). A 0.22 µm syringe filter was used to remove bacteria. Finally, 200 µg of carbenicillin  
84 and penicillin/streptomycin were added to the medium. The final product was stored at 4°C until  
85 use (Fig. 1).

86

#### 87 *Nasonia rearing medium (NRMv2)*

88           Approximately the same number of *S. bullata* pupae were collected as described above.  
89 Pupae were subsequently rinsed in sterile millipore water to remove small particulates. They  
90 were then crushed by hand through a 100 µm nylon mesh and the filtrate was collected in a  
91 sterile glass beaker. Nylon powder-free non-sterile gloves were worn during this extraction. The  
92 filtrate was centrifuged at 4°C (25,000xG) for 10 minutes to separate the sediment, protein, and  
93 lipid layers. Using a 22 gauge needle, the protein layer was transferred to a sterile beaker.

94 Schneider's *Drosophila* media was added to the protein extract to triple the volume and the  
95 resulting mixture was passed through filters with gradually smaller pore sizes (11, 6, 2.5, 0.8,  
96 and 0.45  $\mu\text{m}$ ). A 0.22  $\mu\text{m}$  syringe filter was used to remove bacteria. The final product was  
97 stored at 4°C until use (Fig. 1).

98

#### 99 *Nasonia* strains and collections

100 *N. vitripennis* (strain AsymCx; *Wolbachia* uninfected) mated females were hosted on *S.*  
101 *bullata* pupae and housed in glass culture tubes capped with cotton at  $25 \pm 2^\circ\text{C}$  in constant light,  
102 as previously described (Werren & Loehlin, 2009b). After 10-12 days, *S. bullata* pupariums were  
103 opened and virgin *N. vitripennis* females were collected as pupae from the resulting offspring.  
104 Upon adult eclosion, individual virgin females were isolated and provided two *S. bullata* pupae  
105 for hosting to increase the number of eggs deposited in subsequent hostings. In haplodiploids,  
106 virgin females are fecund and lay all male (haploid) offspring. Two days after initial hostings,  
107 females were provided with a new *S. bullata* pupae housed in a Styrofoam plug, allowing her to  
108 oviposit only on the anterior end of the host for easy embryo collection.

109

#### 110 *Germ-free rearing of Nasonia*

111 *N. vitripennis* strain AsymCx embryos were extracted from *S. bullata* pupae parasitized  
112 by virgin females after 12-24 hours. 20-25 embryos were placed on a 3  $\mu\text{m}$  pore transwell  
113 polyester membrane (Costar, Corning Incorporated, Corning, NY) and sterilized twice with 70  $\mu\text{l}$   
114 10% bleach solution and once with 70  $\mu\text{l}$  70% ethanol solution. The embryos were then rinsed  
115 three times with 80  $\mu\text{l}$  sterile millipore water. After rinsing, the transwell insert was moved into a



116 24 well plate with 250  $\mu$ l of NRM in the well. All plates were stored in a sterile Tupperware box  
117 at  $25 \pm 2^\circ\text{C}$  in constant light conditions for the duration of the experiment. Under sterile laminar  
118 flow, transwells were moved to new wells with 250  $\mu$ l of fresh NRM every second day.  
119 Approximately 1.5 ml of NRM was used per transwell over the duration of the experiment. After  
120 eleven days, the transwells were moved to dry wells in a clean plate and the 12 empty  
121 surrounding wells were filled with 1 ml of sterile millipore water to increase humidity. Two  
122 plates with 12 transwells each (total of 24) were set up using either NRMv1 or NRMv2 for  
123 *Nasonia in vitro* rearing.

124

#### 125 *Comparative analysis of development*

126 A picture was taken of each well, every day for 20 days, under magnification using a  
127 microscope-attached AmScope MT1000 camera. A baseline for the number of larvae present in a  
128 well was determined by counting the number of larvae present in transwell pictures 3 days after  
129 embryo deposition on the transwell membranes (Day 3). Survival estimates were determined by  
130 counting the number of live larvae and pupae on Day 6 and 11, respectively, and compared to  
131 Day 3. Larvae were identified as dead if they were visibly desiccated or malformed. Larval  
132 length was determined using ImageJ software by measuring the anterior to posterior end of  
133 larvae on Days 3, 6, and 14. The proportion of adults produced by a transwell was determined as  
134 follows: (the number of larvae on Day 3 - the number of larvae and pupae remaining on Day 20)  
135  $\div$  the number of larvae on Day 3. Pictures of conventionally reared and germ-free (NRMv2)  
136 adult males were taken, and ImageJ was used to measure head width, which is a correlate for  
137 body size in *Nasonia* (Blaul & Ruther, 2012; Tsai et al., 2014).

139 **RESULTS**

140 Larval growth of *Nasonia vitripennis* reared on NRMv1 was previously compared to  
141 conventionally reared *N. vitripennis* and there were no differences in larval survival or larval  
142 growth over development (Brucker & Bordenstein, 2012a). Here we demonstrate, in  
143 comparisons between NRMv1 and NRMv2, that there is also no difference in larval survival (Fig  
144 2A; Mann-Whitney U (MWU) for Day 6  $p = 0.19$  and Day 14  $p = 0.41$ ) nor length, measured as  
145 the distance from the anterior to posterior end (Fig 2B; MWU for Day 3  $p = 0.26$ , Day 6  $p =$   
146  $0.18$ , Day 14  $p = 0.13$ ). Moreover, a visual comparison of larval sizes on NRMv1 and NRMv2  
147 shows no major differences (Fig 2C-F). These findings indicate that removal of residual bleach,  
148 FBS, and antibiotics does not have a significant impact on larval survival or development.

149 NRMv1 yielded low adult survival compared to conventional rearing (Brucker &  
150 Bordenstein, 2012a). To investigate if using NRMv2 improves larval to adult survival, both the  
151 number of transwells producing adults and the average number of adults produced per transwell  
152 were compared between NRMv1 and NRMv2. The number of transwells that produced at least a  
153 single adult did not differ between NRMv1 (79% productive;  $N=24$ ) and NRMv2 (88%  
154 productive;  $N=24$ ; Fig 3A; Fisher's exact test  $p = 0.7$ ). However and importantly, NRMv2  
155 yielded a higher proportion of adults than NRMv1 (Fig 3B; MWU  $p = 0.001$ ), accounting for a  
156 52% increase in larval to adult survival. Finally, to ensure that adults produced in the *in vitro*  
157 system are similar in size to conventional adults, the head width of adult males produced on  
158 NRMv2 ( $N=16$ ) was compared to conventionally reared ( $N=16$ ) adult males, and there was no  
159 significant difference (Fig 3C; MWU  $p = 0.72$ ).

160

## 161 **DISCUSSION**

162           The previously established *Nasonia in vitro* germ-free rearing protocol (Brucker &  
163 Bordenstein, 2012a), which involved sterilizing embryos and feeding the larvae NRMv1, was  
164 crucial for conducting experiments on *Nasonia*-microbiota interactions (Brucker & Bordenstein,  
165 2013). However, this initial version of the germ-free rearing system contained highly artificial  
166 elements such as bleach rinsing, FBS, and antibiotics (Fig 1; NRMv2). Following removal of  
167 these elements, we show that the alterations to the NRM did not influence larval survival (Fig  
168 2A) or growth (Fig 2B), but importantly resulted in a 52% increase in larval to adult survival  
169 (Fig 3B). Moreover, the size of adult males produced on NRMv2 and *in vivo* do not differ (Fig  
170 3C), suggesting that both *in vitro* and *in vivo* rearing produce morphologically similar adults.

171           Aside from making the *Nasonia in vitro* system more biologically relevant, the new  
172 media has multiple experimental implications. For example, antibiotics are a confounding  
173 variable with unknown consequences to *Nasonia* biology, and they can hinder inoculation  
174 capabilities of the system by causing bacterial communities introduced to rapidly shift in  
175 composition. Thus, removal of antibiotics in NRMv2 makes it easier to derive conclusions and  
176 may provide more flexibility for inoculations *in vitro*, namely introduction of full microbial  
177 communities derived from *Nasonia* species. This new system permits the introduction of both  
178 autochthonous and allochthonous microbial communities, enabling investigations of the  
179 functional relevance of host-specific microbial communities or microbial species. For example,  
180 the *Nasonia* microbiota exhibits "phylosymbiosis", a pattern in which microbial community  
181 relationships parallel the phylogenetic relationships of the host species (Brucker & Bordenstein,

182 2012b; Brucker & Bordenstein, 2013). Transplanting communities between species will test the  
183 functional relevance of phyllosymbiosis.

184 Furthermore, improved survival of larvae to adults on NRMv2 makes obtaining sample  
185 sizes of adults and the measurement of adult phenomes (e.g., physiology, anatomy, and behavior)  
186 more feasible. In this context, NRMv2 permits improved exploration of *Nasonia* adult-  
187 microbiota interactions. For example, there are many examples of microbe-mediated signals used  
188 in mate-choice, species recognition, and kin recognition (Reviewed in Shropshire & Bordenstein,  
189 2016). *Nasonia* species produce several different signals including cuticular hydrocarbons  
190 (Buellesbach et al., 2013), abdominal sex pheromones (Diao et al., 2016), and cephalic  
191 pheromones housed in an oral gland (Miko & Deans, 2014; Ruther & Hammerl, 2014). This *in*  
192 *vitro* rearing system allows for the exploration of the interaction of microbes with host signals to  
193 test what role these complex interactions may have in adult behavior, insect communication, and  
194 reproductive isolation.

195 Parasitoid wasps are also difficult to study developmentally because the fly host's  
196 puparium obstructs visualization of the *Nasonia* larvae and pupae, preventing multiple measures  
197 of a single individual over time. *In vitro* rearing of *Nasonia* allows for observations of single  
198 individuals over developmental time and for strict control of larval diet, bacterial exposure, and  
199 *Nasonia* density. Using this system, one may test how these variables influence metamorphosis  
200 (Johnston & Rolff, 2015), wing and body size (Rivers & Losinger, 2014), craniofacial anomalies  
201 (Werren et al., 2015), and many other physiological traits.

202 In summary, we have streamlined and improved upon the *Nasonia in vitro* rearing system  
203 while removing antibiotics and other factors from the equation. These changes open the door to

204 multidisciplinary studies of host-microbiota interactions and development and add to *Nasonia*'s  
205 utility as a model system.

206

207

## 208 **PROTOCOL**

### 209 *Preparing Nasonia Rearing Medium*

210 1. Fill a sterilized beaker with 150 ml of *S. bullata* pupa. Remove larvae, poor quality  
211 pupae, and debris.

212 2. In the beaker, cover pupae with sterile water, allow to sit for 1 min, and strain to remove  
213 surface particulates from the puparium surface. Note: Some moisture will remain on the  
214 pupae.

215 3. Crush the pupae by hand (covered with powder-free nitrile gloves) and squeeze juices  
216 through a 100  $\mu\text{m}$  nylon mesh to remove the *S. bullata* puparium.

217 4. Pour juices (approximately 70-90 ml) into a conical tube and seal tightly.

218 5. Centrifuge the mixture for 10 min at 4°C (25,000xG). The mixture will separate into three  
219 distinct layers: a sediment, protein, and lipid layer from bottom to top, respectively.

220 6. To prevent clogging during filtration, extract the protein layer using a 22 gauge sterile  
221 needle and transfer it to a sterile beaker under sterile laminar flow.

222 7. Add a 2:1 ratio of Schneider's *Drosophila* medium to the protein extract.

223 8. Filter the media through progressively smaller pore sizes (11, 6, 2.5, 0.8, and 0.45  $\mu\text{m}$   
224 filters) to remove increasingly smaller particulates. To prevent clogging, replace filter  
225 when flow begins to slow.

- 226 9. Sterilize the media by filtering through a 0.22  $\mu\text{m}$  filter, taking care to use aseptic  
227 technique.
- 228 10. Store at 4°C for up to 2 weeks.
- 229 11. Filter NRM through a 0.22  $\mu\text{m}$  filter before use to ensure sterility and remove  
230 sedimentation.
- 231

## 232 REFERENCES

- 233 Ashmead WH, Smith HH. 1904. *Classification of the Chalcid Flies: Or the Superfamily*  
234 *Chalcidoidea, with Descriptions of New Species in the Carnegie Museum, Collected in*  
235 *South America by Herbert H. Smith*: authority of the Board of Trustees of the Carnegie  
236 Institute.
- 237 Baeder JM, King BH. 2004. Associative learning of color by males of the parasitoid wasp  
238 *Nasonia vitripennis* (Hymenoptera : Pteromalidae). *Journal of Insect Behavior* 17:201-  
239 213. DOI: 10.1023/B:JOIR.0000028570.93123.dc
- 240 Beukeboom LW, van den Assem J. 2001. Courtship and mating behaviour of interspecific  
241 *Nasonia* hybrids (Hymenoptera, Pteromalidae): a grandfather effect. *Behavioral Genetics*  
242 31:167-177.
- 243 Blaul B, Ruther J. 2012. Body size influences male pheromone signals but not the outcome of  
244 mating contests in *Nasonia vitripennis*. *Animal Behaviour* 84:1557-1563. DOI:  
245 10.1016/j.anbehav.2012.10.001
- 246 Bordenstein SR, O'Hara FP, Werren JH. 2001. Wolbachia-induced incompatibility precedes  
247 other hybrid incompatibilities in *Nasonia*. *Nature* 409:707-710. DOI: 10.1038/35055543
- 248 Bordenstein SR, Werren JH. 2007. Bidirectional incompatibility among divergent *Wolbachia*  
249 and incompatibility level differences among closely related *Wolbachia* in *Nasonia*.  
250 *Heredity* 99:278-287. DOI: 10.1038/sj.hdy.6800994
- 251 Brucker RM, Bordenstein SR. 2012a. In vitro cultivation of the hymenoptera genetic model,  
252 *Nasonia*. *PLoS One* 7:e51269. DOI: 10.1371/journal.pone.0051269
- 253 Brucker RM, Bordenstein SR. 2012b. Speciation by symbiosis. *Trends in Ecology & Evolution*  
254 27:443-451. DOI: 10.1016/j.tree.2012.03.011
- 255 Brucker RM, Bordenstein SR. 2013. The hologenomic basis of speciation: gut bacteria cause  
256 hybrid lethality in the genus *Nasonia*. *Science* 341:667-669. DOI:  
257 10.1126/science.1240659
- 258 Buellesbach J, Gadau J, Beukeboom LW, Echinger F, Raychoudhury R, Werren JH, Schmitt T.  
259 2013. Cuticular hydrocarbon divergence in the jewel wasp *Nasonia*: evolutionary shifts in  
260 chemical communication channels? *Journal of Evolutionary Biology* 26:2467-2478. DOI:  
261 10.1111/jeb.12242
- 262 Clark ME, O'Hara FP, Chawla A, Werren JH. 2010. Behavioral and spermatogenic hybrid male  
263 breakdown in *Nasonia*. *Heredity* 104:289-301. DOI: 10.1038/hdy.2009.152
- 264 Davies NJ, Tauber E. 2015. WaspAtlas: a *Nasonia vitripennis* gene database and analysis  
265 platform. *Database (Oxford)* 2015. DOI: 10.1093/database/bav103
- 266 Diao W, Mousset M, Horsburgh GJ, Vermeulen CJ, Johannes F, van de Zande L, Ritchie MG,  
267 Schmitt T, Beukeboom LW. 2016. Quantitative Trait Locus Analysis of Mating Behavior  
268 and Male Sex Pheromones in *Nasonia* Wasps. *G3: Genes| Genomes| Genetics*. DOI:  
269 10.1534/g3.116.029074
- 270 Drapeau MD, Werren JH. 1999. Differences in mating behaviour and sex ratio between three  
271 sibling species of *Nasonia*. *Evolutionary Ecology Research* 1:223-234.
- 272 Ferree PM, Avery A, Azpurua J, Wilkes T, Werren JH. 2008. A bacterium targets maternally  
273 inherited centrosomes to kill males in *Nasonia*. *Current Biology* 18:1409-1414. DOI:  
274 10.1016/j.cub.2008.07.093
- 275 Honn KV, Singly JA, Chavin W. 1975. Fetal Bovine Serum: A multivariate standard.  
276 *Proceedings of the society for experimental biology and medicine* 149:344-347.

277 Johnston PR, Rolff J. 2015. Host and Symbiont Jointly Control Gut Microbiota during Complete  
278 Metamorphosis. *PLoS Pathology* 11:e1005246. 10.1371/journal.ppat.1005246  
279 Lynch JA. 2015. The expanding genetic toolbox of the wasp *Nasonia vitripennis* and its  
280 relatives. *Genetics* 199:897-904. DOI: 10.1534/genetics.112.147512  
281 Miko I, Deans A. 2014. The mandibular gland in *Nasonia vitripennis* (Hymenoptera:  
282 Pteromalidae). *bioRxiv*. DOI: 10.1101/006569  
283 Raychoudhury R, Desjardins CA, Buellesbach J, Loehlin DW, Grillenberger BK, Beukeboom L,  
284 Schmitt T, Werren JH. 2010. Behavioral and genetic characteristics of a new species of  
285 *Nasonia*. *Heredity* 104:278-288. 10.1038/hdy.2009.147  
286 Rivers DB, Losinger M. 2014. Development of the gregarious ectoparasitoid *Nasonia vitripennis*  
287 using five species of necrophagous flies as hosts and at various developmental  
288 temperatures. *Entomologia experimentalis et applicata* 151:160-169. DOI:  
289 10.1111/eea.12182  
290 Ruther J, Hammerl T. 2014. An oral male courtship pheromone terminates the response of  
291 *Nasonia vitripennis* females to the male-produced sex attractant. *Journal of Chemical*  
292 *Ecology* 40:56-62. DOI: 10.1007/s10886-013-0372-2  
293 Shropshire JD, Bordenstein SR. 2016. Speciation by Symbiosis: the Microbiome and Behavior.  
294 *MBio* 7. DOI: 10.1128/mBio.01785-15  
295 Steiner S, Hermann N, Ruther J. 2006. Characterization of a female-produced courtship  
296 pheromone in the parasitoid *Nasonia vitripennis*. *Journal of Chemical Ecology* 32:1687-  
297 1702. DOI: 10.1007/s10886-006-9102-3  
298 Tsai Y-JJ, Barrows EM, Weiss MR, Zeh D. 2014. Pure Self-Assessment of Size During Male-  
299 Male Contests in the Parasitoid Wasp *Nasonia vitripennis*. *Ethology* 120:816-824. DOI:  
300 10.1111/eth.12254  
301 Verhulst EC, Lynch JA, Bopp D, Beukeboom LW, van de Zande L. 2013. A new component of  
302 the *Nasonia* sex determining cascade is maternally silenced and regulates transformer  
303 expression. *PLoS One* 8:e63618. DOI: 10.1371/journal.pone.0063618  
304 Werren JH, Cohen LB, Gadau J, Ponce R, Baudry E, Lynch JA. 2015. Dissection of the complex  
305 genetic basis of craniofacial anomalies using haploid genetics and interspecies hybrids in  
306 *Nasonia* wasps. *Developmental Biology*. 10.1016/j.ydbio.2015.12.022  
307 Werren JH, Loehlin DW. 2009a. Rearing *Sarcophaga bullata* fly hosts for *Nasonia* (parasitoid  
308 wasp). *Cold Spring Harbor Protocols* 2009:pdb prot5308. DOI: 10.1101/pdb.prot5308  
309 Werren JH, Loehlin DW. 2009b. Strain maintenance of *Nasonia vitripennis* (parasitoid wasp).  
310 *Cold Spring Harbor Protocols* 2009:pdb prot5307. DOI: 10.1101/pdb.prot5307  
311 Zwier MV, Verhulst EC, Zwahlen RD, Beukeboom LW, van de Zande L. 2012. DNA  
312 methylation plays a crucial role during early *Nasonia* development. *Insect Molecular*  
313 *Biology* 21:129-138. DOI: 10.1111/j.1365-2583.2011.01121.x



## Figure 1 (on next page)

Figure 1

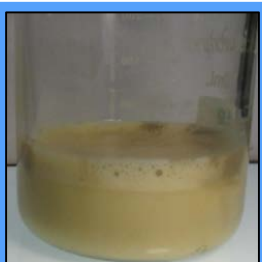
**Schematic of the workflow to produce *Nasonia* Rearing Media (NRM).** Red boxes indicate steps present in NRMv1 but eliminated in NRMv2. Blue boxes indicate steps present in both procedures. Pictures to the right show the visual progression from *S. bullata* pupae to final NRM product. L, lipid layer; P, protein layer; S, sediment layer.

Collect *S. bullata* pupae



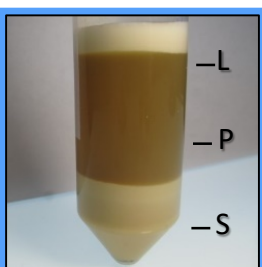
Rinse with bleach

Rinse with sterile H<sub>2</sub>O



Crush and collect fluids

Centrifuge and extract protein



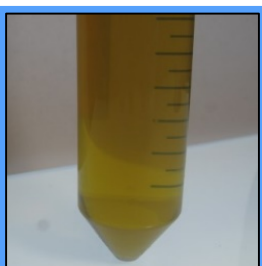
Add Schneider's *Drosophila* media

Add fetal bovine serum



Pre-filter

Filter sterilize with 0.22 μm filter



Add antibiotics

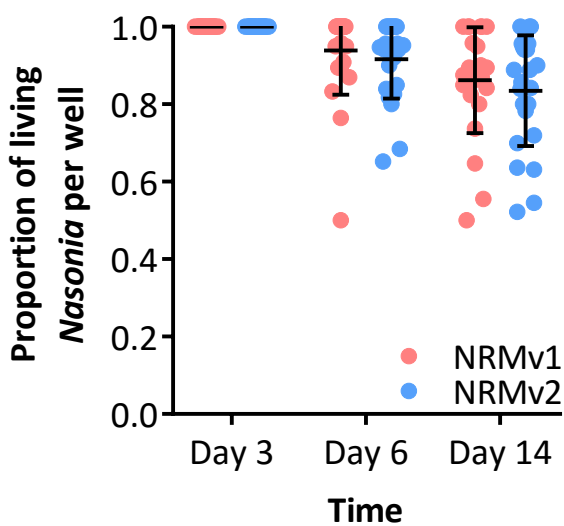
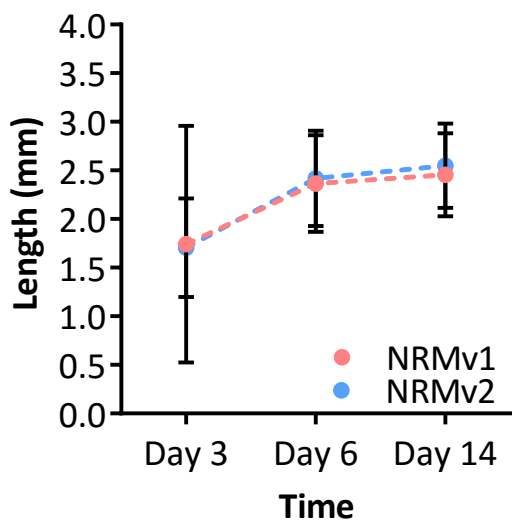
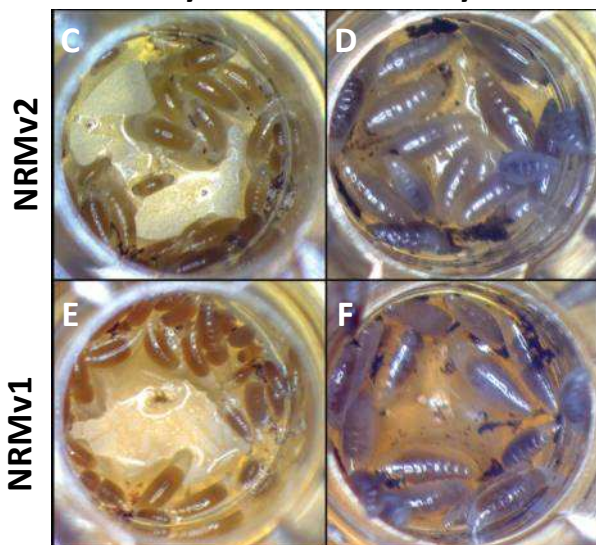
Store at 4°C until use

## **Figure 2**(on next page)

Figure 2

### **Comparison of *Nasonia* germ-free larval development on NRMv1 and NRMv2. (A)**

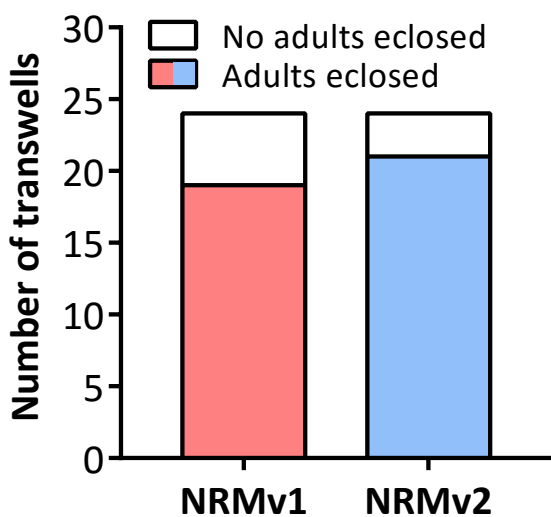
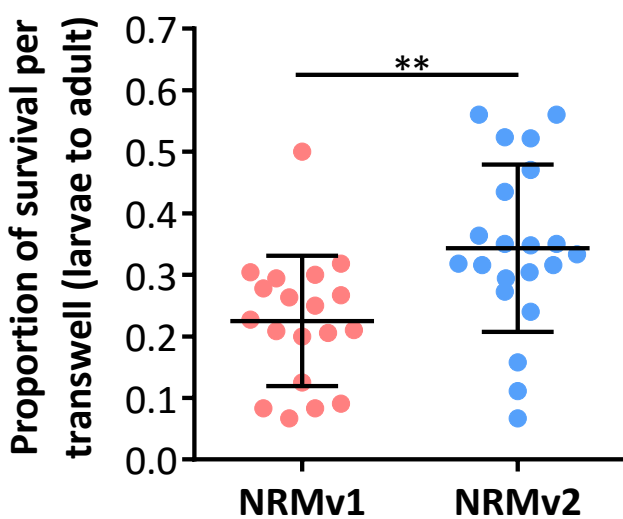
The number of living *Nasonia vitripennis* in transwells on Days 3, 6, and 14. There are no statistically significant differences in larval survival on NRMv1 and NRMv2. (B) Equivalent larval lengths measured from anterior to posterior end in mm. (C-F) Visual comparison of larvae reared on NRMv1 and NRMv2 on Days 6 and 9. Vertical bars with caps represent standard deviation from the mean.

**A****B****Day 3****Day 9**

## Figure 3 (on next page)

Figure 3

**Survival and size of *Nasonia* germ-free adult males.** (A) Comparison of the number of transwells producing adults between NRMv1 and NRMv2. (B) Proportion of larval to adult survival in each transwell is determined as follows: (the number of larvae on Day 3 - the number of larvae and pupae remaining on Day 20) ÷ the number of larvae on Day 3. (C) Adult head widths from germ-free males reared on NRMv2 and males reared conventionally. Larval to adult survival was statistically different between the two media (Mann-Whitney U, P-value = 0.001). All other measures were not significant with  $\alpha = 0.05$ . Vertical bars with caps represent standard deviation from the mean.

**A****B****C**