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TECHNOLOGY REPORT

Oikopleura dioica Culturing Made Easy: A Low-Cost Facility for an Emerging Animal Model in EvoDevo

Josep Martí-Solans,[1] Alfonso Ferrández-Roldán,[1] Hector Godoy-Marín,[1] Jordi Badia-Ramentol,[1] Nuria P. Torres-Aguila,[1] Adriana Rodríguez-Marí,[1] Jean Marie Bouquet,[2,3] Daniel Chourrout,[2] Eric M. Thompson,[2,3] Ricard Albalat,[1]* and Cristian Cañestro[1]*

[1]Departament de Genètica and Institut de Recerca de la Biodiversitat (IRBio), Universitat de Barcelona, Barcelona, 08028, Spain

[2]Sars International Centre for Marine Molecular Biology, University of Bergen, N-5008 Bergen, Bergen, Norway

[3]Department of Biology, University of Bergen, Postbox 7803, N-5020 Bergen, Norway

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Summary: The genome sequencing and the development of RNAi knockdown technologies in the urochordate *Oikopleura dioica* are making this organism an attractive emergent model in the field of EvoDevo. To succeed as a new animal model, however, an organism needs to be easily and affordably cultured in the laboratory. Nowadays, there are only two facilities in the world capable to indefinitely maintain *Oikopleura dioica*, one in the SARS institute (Bergen, Norway) and the other in the Osaka University (Japan). Here, we describe the setup of a new facility in the University of Barcelona (Spain) in which we have modified previously published husbandry protocols to optimize the weekly production of thousands of embryos and hundreds of mature animals using the minimum amount of space, human resources, and technical equipment. This optimization includes novel protocols of cryopreservation and solid cultures for long-term maintenance of microalgal stocks—*Chaetoceros calcitrans*, *Isochrysis sp.*, *Rhinomonas reticulate*, and *Synechococcus sp.*—needed for *Oikopleura dioica* feeding. Our culture system maintains partially inbred lines healthy with similar characteristics to wild animals, and it is easily expandable to satisfy on demand the needs of any laboratory that may wish to use *Oikopleura dioica* as a model organism. genesis 53:183–193, 2015. © 2014 Wiley Periodicals, Inc.

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In the last decades, the study of well-established animal models has been crucial for the extraordinary progress of knowledge in the fields of developmental biology and biomedicine, but the scientific challenges of the XXI century, especially those to decipher the evolutionary basis of life diversity, demand the development of new animal models carefully chosen according to their phylogenetic position in the tree of life (Maher, 2009). The larvacean (or appendicularian) *Oikopleura dioica*, for instance, is a planktonic marine organism that

Additional Supporting Information may be found in the online version of this article.

Alfonso Ferrández-Roldán, Hector Godoy-Marín, Jordi Badia-Ramentol, and Nuria P. Torres-Águila contributed equally to this work.

Current Address for Adriana Rodríguez-Marí: Institut de Bioenginyeria de Catalunya, IBEC, Barcelona, 08028, Spain.

*Correspondence to: Ricard Albalat, Departament de Genètica and Institut de Recerca de la Biodiversitat (IRBio), Universitat de Barcelona, Barcelona, 08028, Spain. E-mail: ralbalat@ub.edu (or) Cristian Cañestro, Departament de Genètica and Institut de Recerca de la Biodiversitat (IRBio), Universitat de Barcelona, Barcelona, 08028, Spain. E-mail: canestro@ub.edu

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possesses many characteristics that make it attractive as animal model: (*i*) *O. dioica* occupies a key phylogenetic position within the chordate phylum as a basally divergent member of the urochordate subphylum (Stach and Turbeville, 2002; Swalla *et al.*, 2000; Wada, 1998), which is the sister group of vertebrates (Delsuc *et al.*, 2006; Delsuc *et al.*, 2008). *O. dioica*, therefore, is a useful model to infer the ancestral condition from which vertebrate and other urochordate species evolved. (*ii*) The genome of *O. dioica* is very small (~70 Mb) and appears to have suffered an extreme process of compaction leading to very small introns (peak at 47 bp) and intergenic regions (53% < 1 kb), and absence of most pan-animal transposable elements (Cañestro and Albalat, 2012; Chavali *et al.*, 2011; Denoeud *et al.*, 2010). This drastic genome compaction has been accompanied by an extreme shattering of exon–intron organization and physical gene reordering, and massive gene losses accompanied by extensive lineage specific gene duplications. Despite this deep reorganization of the genome, *O. dioica* conserves a typical chordate body plan, which makes this organism an interesting model of extreme genome plasticity and body plan conservation, *a.k.a*. the "inverse paradox of Evo-Devo" (Cañestro *et al.*, 2007; Denoeud *et al.*, 2010). (*iii*) *O. dioica* has a short generation time of just 5 days at 20°C (Fenaux, 1998) (comparable to *C. elegans*) and a high fecundity with hundreds of eggs per female that can be externally fertilized in vitro. *O. dioica* provides an excellent animal model for studies in developmental biology, as fertilized eggs develop into transparent embryos easy to follow under a stereomicroscope without any further manipulation. Many developmental studies have already described its early embryogenesis (Delsman, 1910; Nishida, 2008), its stereotyped cell fate map (Fujii *et al.*, 2008; Stach *et al.*, 2008), as well as the development of several body structures such as the notochord (Bassham and Postlethwait, 2000; Nishino *et al.*, 2001), the central nervous system (Cañestro *et al.*, 2005; Cañestro and Postlethwait, 2007; Soviknes *et al.*, 2007), placodal sensory organs (Bassham and Postlethwait, 2005), the digestive system (Burighel and Brena, 2001; Cañestro *et al.*, 2010), gonad development (Ganot *et al.*, 2007; Ganot *et al.*, 2008), or the homolog of the thyroid (Bassham *et al.*, 2008; Cañestro *et al.*, 2008). *iv*). Finally, recent technical advances in RNAi gene silencing by injecting dsRNA into the female gonad has definitively opened the possibility to make this animal a model for functional studies gaining new insights into genes of our interest (Omotezako *et al.*, 2013).

Despite *O. dioica* is cosmopolitan and widely available around the globe, the design of an easy and affordable system to culture *O. dioica* in any laboratory interested in studying this organism remains a challenge. Breeding of *O. dioica* in the laboratory was already successfully achieved 40 years ago (Paffenhöfer, 1973) and since then, several laboratories have tried protocols for its husbandry during a limited number of generations by mixing mature male and female specimens and let them spontaneously spawn in beakers in which animals were maintained in suspension by different devices designed *ad hoc* (Bassham and Postlethwait, 2000; Chioda *et al.*, 2002; Fenaux and Gorsky, 1979, 1985; Lopez-Urrutia and Acuña, 1999; Nishino *et al.*, 2000; Sato *et al.*, 1999; Troedsson et al., 2013). Currently, however, to our knowledge there are only two active laboratories with animal facilities capable to indefinitely maintain *O. dioica* cultures all year around, one in the SARS Institute in Bergen (Norway) (Bouquet *et al.*, 2009) and another in the Osaka University (Japan) (Nishida, 2008). These animal facilities have the capability to reliably produce large amounts of adult animals and embryos every week, but they require also large amounts of laboratory space, seawater, microalgal cultures, equipment, and human resources, which make their setups unaffordable for most standard research laboratories. With the aim of simplifying and scaling down the *O. dioica* husbandry system, we have modified and optimized the conditions described for the existing facilities. Despite the animal production is lower in our system, our husbandry protocol is flexible enough to provide the biological material to satisfy the requirements for most standard research groups using the minimum amounts of space, technical equipment, laboratory expenses, and human resources.

METHODS AND RESULTS

Animal Collection, Facility, and Seawater

Adult animals were collected in three different locations of the Catalonian coast near Barcelona (Fig. 1a; see Supporting Information Methods for details) and raised in our animal facility, which was inspirited on the *Oikopleura* culture facilities described in Nishida 2008 and Bouquet *et al.* 2009, but modified to satisfy our experimental requirements. Our animal facility was set in a small room (about 5 m^2) maintained at 19 ± 1°C using a standard air conditioner device. Animals were maintained in seawater filtered through 50 μm and 20 μm polypropylene filters (MBHP050-10 and LTPPBB020-10 AscoFiltri Filtering cartridges, respectively) (Fig. 1b; see Supporting Information Methods for details) and feed with microalgae. For in vitro fertilization experiments and for microalgal cultures, filtered seawater (fSW) was sterilized (sterilized seawater, sSW) using 0.22 μm filters (VacuCap PF Filters 4622, Pall Corporation) (Albalat et al., 2003; Dalfó *et al.*, 2001).

Microalgal Production for *O. dioca* Feeding

The diet for *O. dioica* consisted of four microalga species, three true eukaryotic species of microalga, *Chaetoceros calcitrans* (CCAP 1010/11), *Isochrysis sp*. (CCAP

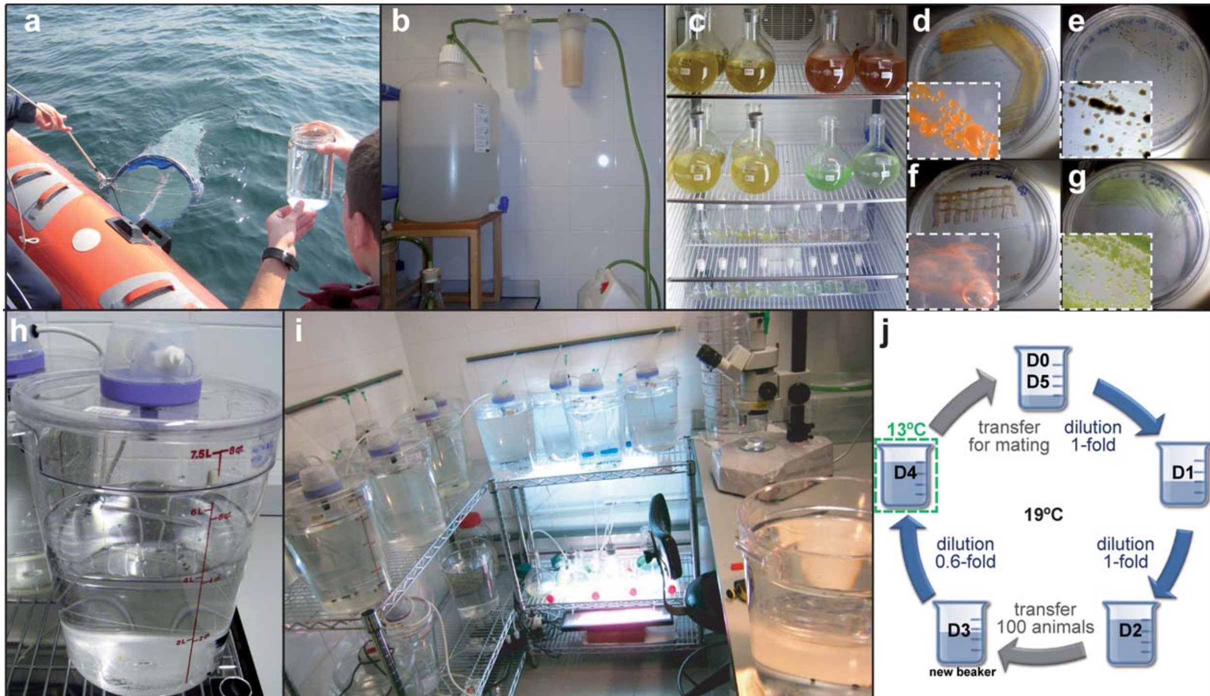

FIG. 1. *O. dioica* facility in the University of Barcelona (Catalonia, Spain). (**a**) Animals were collected in the coast of Catalonia near Barcelona using a plankton net or directly with a bucket. (**b**) Seawater is filtrated at 50–20 μm (fSW) in the facility to remove excess of sand particles that could affect *O. dioica* buoyancy. (**c**) The production of the four microalgae for *O. dioica* feeding (Bouquet *et al*., 2009) was scale down in an adaptable fashion to the weekly needs of the facility (round-bottom glass flasks in upper shelves). Long-term stocks (100 mL Erlenmeyers in lower shelves) were renewed just once per month. (**d-g**) The use of agar plates provides an alternative method to maintain long-term microalgal stocks: (**d**) *Isochrysis sp*., (**e**) *Chaetoceros calcitrans*, (**f**) *Rhinomonas reticulata* and (**g**) *Synechococcus sp*. Solid cultures also allow recovering isolated colonies (insets **d-g**), which can be useful in case of contamination. *R. reticulata* grow better within the agar matrix rather on the surface (notice the bubble in inset **f**), and despite colonies are not easily formed, individual single cells are easily observed. (**h**) *O. dioica* animals were maintained in suspension by the rotation of a paddle driven by a motor mounted on the lid of polycarbonate beakers. (**i**) Animal lines were maintained in a small room (5 m^2) in four shelves (1.5 m^2) at 19°C using a standard air conditioner device. (**j**) The protocol of husbandry has been designed to make a life cycle of five days at 19°C in which each generation start with a mating of 10–15 males and 20–30 females close to spawn (D0). The next two days (D1 and D2) cultures are just diluted onefold (1/2 dilution) with fresh fSW, and at the third day (D3), 100 animals are transferred to a new beaker with fresh fSW. At day 4 (D4), cultures are 0.6-fold diluted (2/3 dilution) with fresh fSW and moved to a 13°C incubator. In the morning of day 5 (D5), most animals synchronously become mature, and are ready to set a new mating for next generation. This protocol of husbandry has been optimized to maximize the reduction of the amount of seawater, number of beakers, volume of microalgae feeding cultures, and tasks of manipulation. This protocol of husbandry is easily expandable on demand (i.e., by increasing the number of matings on D0 or the number of transfers on D3), which allows us to easily generate hundreds of mature animals and tens of thousands of embryos every week.

927/14), *Rhinomonas reticulata* (CCAP 995/2) and one prokaryotic species of cyanobacteria (a.k.a. blue-green algae) *Synechococcus sp*. (K0408), grown in modified Conway medium (Bouquet *et al*., 2009) until their optical density (OD) at 600 nm ranged from 0.20 to 0.25 (8×10^6 to 1×10^7 cells/ml) for *C. calcitrans*, from 0.13 to 0.18 (2×10^6 to 3×10^6 cells/ml) for *Isochrysis sp*., from 0.14 to 0.20 (1×10^6 to 1.5×10^6 cells/ml) for *R. reticulata* and from 0.15 to 0.20 (4.5×10^7 to 6×10^7 cells/ml) for *Synechococcus sp*. (Fig. 1c; see Supporting Information Methods for details).

Long-Term Maintenance of Microalgal Strain Stocks

The maintenance of healthy stocks of the four microalgal strains is crucial for the culturing system of *O. dioica*. This maintenance, however, is a time-consuming task that requires weekly renewal and is sensitive to contamination. To optimize this task, we designed three strategies that minimized the manipulation of the stocks and reduced the risk of contamination: (*i*) long-term liquid cultures; (*ii*) long-term solid cultures; and (*iii*) cryopreserved microalgae stocks.

Long-term liquid cultures. Long-term cultures were generated by finding the inoculum with minimum number of cells that guaranteed the recovery of the culture but delayed the reach of the declining phase as much as possible. We empirically determined that inocula containing OD-estimates of $\approx 10^2$ cells for *R. reticulata*, $\approx 10^3$ cells for *C. calcitrans* and *Isochrysis sp*., and $\approx 10^4$ cells for *Synechococcus sp*. in 50 mL of modified Conway medium were enough to guarantee the recovery

of the cultures and reduced the renewal to just once per month, when their OD reached 0.35–0.60 (Fig. 1c and for details see Supporting Information Fig. 1).

Long-term solid cultures. The second strategy consisted in culturing the microalgae in agar plates that could be maintained in good conditions for long periods, at the same time that allowed the isolation of individual colonies in case of accidental contamination. Agar (Pronadisa #1800.00) was dissolved in modified Conway medium by autoclaving, and vitamins were added to cooled media before agar solidified. The streaks were made from the pellet from 5 mL of a microalgal liquid culture (OD > 0.25) centrifuged for 5 min at 756 g (2,500 rpm). Plates were incubated upside-down at 13°C with 12 h photoperiod until microalgal colonies became apparent after 1–2 weeks (Fig. 1d–g). Agar concentration appeared to be a critically variable for the growth of each microalga, and we found that *Isochrysis sp*. grew well in concentrations from 1.5% to 0.5%, *C. calcitrans* from 1% to 0.5%, whereas *Synechococcus sp* and *R. reticulata* grew better at 0.5% agar and 0.3%, respectively. We have used microalgal colonies plated for at least 3 months to successfully inoculate new plates or liquid medium.

Microalgae cryopreservation. With the exception of *C. calcitrans* (Salas-Leiva, 2011), we did not found specific protocols designed for the cryopreservation of the microalgae that we use for *O. dioica* feeding, and therefore, we modified the conditions of some general protocols that used dimethyl sulfoxide (DMSO) as cryoprotectant (Day, 2007). To cryopreserve our microalgae, 1 mL of densely growth culture (i.e., OD > 0.25 for *Isochrysis sp*. and *R. reticulata;* OD > 0.35 for *C. calcitrans*, and OD > 0.4 for *Synechococcus sp*.) was gently mixed with 1 mL of a DMSO (D8148, Sigma) stock prepared at different concentrations in sSW for each microalga–30%v/v in sSW for *R. reticulata* and *Isochrysis sp*., 20% for *C. calcitrans*, and 10% for *Synechococcus sp*.–in a cryotube (479–6847 Cryo Tube, Nunc Thermo Scientific, VWR), incubated at 19°C during 45 min for *Isochrysis sp*. and *R. reticulata*, 15 min in darkness for *C. calcitrans*, and 5 min for *Synechococcus sp*., transferred into Mr Frosty™ device (5100-001, Nalgene) and incubated in a −80°C ultrafreezer during at least 3 h, and finally stored into liquid nitrogen until used. For culture recovering, cryopreserved microalgae were thawed in a 40°C water-bath during 1–2 min, until ice crystals melted, transferred into 50 mL of modified Conway medium in a 100 mL Erlenmeyer and maintained at 13°C with a 12 h photoperiod. Typically, after 2–3 weeks, microalgal growth was obvious by eye and spectrophotometrically with an OD > 0.1 (Supporting Information Fig. 2). We have shown so far that microalgal cryopreserved stocks were viable at least after 3 months of storage in liquid nitrogen, although additional experiments are needed to validate the quality of the cryopreserved stocks after very long-term storage (i.e., years).

***O. dioica* culturing and mating strategy for line maintenance.** Animals captured from the coast of Barcelona were transferred to fSW in 8-liter polycarbonate beakers (Camwear Round RFSCW8, Cambro) using the large opening of 10 mL plastic pipettes (900036, Deltalab). We added 2–3 pearls of 1-Hexadecanol (258741-500G, Sigma) to each beaker to reduce the surface tension, and 10 g of prerinsed activated charcoal pellets (22631.293 charcoal 0.85–1.7 mm gradient, VWR) to each beaker to preserve water quality, with the exception of the mating beakers in order to not affect embryo development. Animals were maintained in suspension by the rotation of a methacrylate paddle (10 cm × 23 cm) driven at 5–6 rpm by an electric motor (Synchronous motor, Kelvin) mounted on the beaker lid (Fig. 1h,i).

To start an *O. dioica* line (day 0: D0), 20–30 mature females and 10–15 mature males were transferred into a mating beaker with 1.5 liters of fSW at 19°C (Fig. 1j) in which 10 mL of *Isochrysis sp*., 1 mL of *C. calcitrans*, 5 mL of *R. reticulate*, and 5 mL of *Synechococcus sp*. from the 800 mL microalgal cultures had been added. In the next few hours, animals spawned, generating a progeny of thousands of individuals. Next day morning (day 1: D1), one and a half liter of fresh fSW was gently added up to a final volume of 3 liters (1/2 dilution), and animals were fed with 10 mL of *Isochrysis sp*., 2 mL of *C. calcitrans* and 10 mL of *Synechococcus sp*. In the morning of day 2 (D2), 3 liters of fresh fSW were gently added, up to a final volume of 6 liters (1/2 dilution), and animals were fed with 20 mL of *Isochrysis sp*., 4 mL of *C. calcitrans*, 10 mL of *R. reticulate*, and 10 mL of *Synechococcus sp*. At day 3 (D3), from the hundreds of animals available, 100 individual of approximately the same size (to promote that they will mature at the same time) were manually transferred to a new beaker containing 4 liters of fresh fSW, and fed with 20 mL of *Isochrysis sp*., 8 mL of *C. calcitrans*, and 20 mL of *R. reticulata*. At this point, the setting of additional 100-animal beakers allows to scale up on demand the *O. dioica* culture. The beaker with the remaining animals can be maintained as a "backup" population by daily feeding it with 20 mL of *Isochrysis sp*., 8 mL of *C. calcitrans*, 20 mL of *R. reticulate*, and 10 mL of *Synechococcus sp*. At day 4 (D4), two liters of fSW were added gently to each 100-animals culture up to a final volume of 6 liters (2/3 dilution), fed with 30 mL of *Isochrysis sp*., 8 mL of *C. calcitrans*, and 30 mL of *R. reticulata* and incubated at 13°C to slow down the maturation of the gonads. In the morning of day 5 (D5, typically from 9:00 AM to 12:00 PM), most animals were sexually mature and one (or more) new cultures (D0) were started by mating again 20–30 females and 10–15 males.

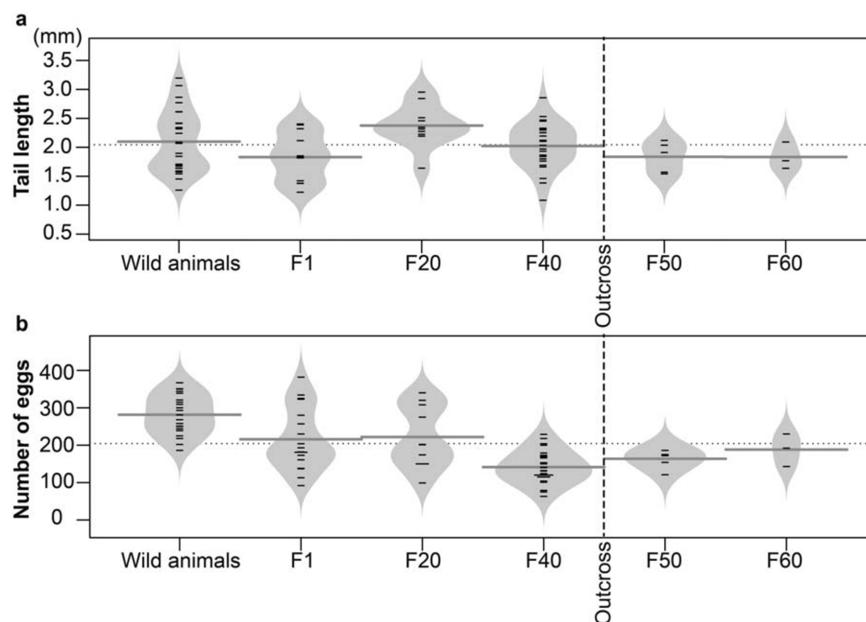

FIG. 2. Analysis of tail lengths (**a**) and number of eggs (**b**) to test the quality and fecundity of inbred populations in the *O. dioica* facility. Tail lengths of mature females and number of eggs of individual spawns are represented as short horizontal lines in one-dimension scatter beanplot (some lines appear bigger because of the coincidence of values in different measurements). Grey areas represent the density distribution of the values, and the thick grey lines indicate the average of tail lengths or number of eggs within each generation. The horizontal dotted line shows the overall average of tail length or number of eggs. The tail length and number of eggs for inbred animals for 1 generation (F1, number of analyzed females $n = 12$), more than 20 (F20, $n = 10$), 40 (F40, $n = 27$), 50 (F50, $n = 5$) or 60 (F60, $n = 5$) generations were compared with those of wild animals ($n = 27$). No significant differences were observed in the length of the tails between inbred animals and wild animals (ANOVA: P-value = 0.061). Comparison of the clutch size was not significantly different between wild animals and inbred animals during the first 20 generations (see Table S1 for statistical significance). At F40, however, the average of the clutch size in some *O. dioica* inbred lines decreased significantly. We then generated an hybrid population by outcrossing two lines independently maintained in the facility (dashed line), and we observed that although the clutch size after the outcross did not significantly improve, the outcross resulted in similar clutch sizes during at least the next 20 generations. The data suggest, therefore, that the inbreeding generated during our husbandry strategy did not affect the average size of animals, and that outcrossing lines can help to limit the reduction of the fecundity observed after many generations of inbreeding. In any case, the overall egg production of our animal facility produced clutch sizes big enough (170 ± 60 eggs in average) to fulfill the experimental needs.

Quality and stability of the *O. dioica* culture through generations. To test the quality and the stability of our *O. dioica* cultures over time, we compared size, fecundity, and fertility parameters between wild animals and inbred animals in our animal facility for 1 (F1) or more than 10 (F10), 20 (F20), 40 (F40), 50 (F50) or 60 (F60) generations. Specifically, the parameters analyzed were the tail length of mature females, the number of eggs per spawn, and the success of fertilization and embryo development up to the hatch. To discard that differences between wild and inbred animals were because of environmental factors (e.g., temperature or food differences in the sea *vs*. our facility) rather than inbreeding or artificial selection derived from our mating strategy, we generated a first generation from wild collected animals maintained in different beakers and raised under our husbandry system. Genetically unrelated animals coming from different beakers were crossed, and the size, fecundity, and fertility values of the born animals were taken as representative measures of those parameters for genetically nonrelated "wild" animals raised under normalized environmental laboratory conditions.

For size comparison, we measured the tails of mature females randomly selected from each group of animals (i.e., wild animals and animals from F1 to F60 generations of inbreeding; $n \geq 10$). Animals were photographed after natural spawning using a Nikon Coolpix E995 camera in a Leica Wild M3Z Kombistereo microscope, and the sizes of their tails were measured with the ImageJ software (http://imagej.nih.gov/ij/). The average and standard deviation of the tail lengths of mature females through different generations F1, F20, F40, F50, and F60 were 1.8 mm ± 0.4 mm, 2.4 mm ± 0.4 mm, 2.0 mm ± 0.4 mm, 1.8 mm ± 0.3 mm, and 1.8 mm ± 0.2 mm, respectively, which were not significantly different among them, neither to the lengths of nonrelated wild animals, 2.1 mm ± 0.5 mm (ANOVA: P-value = 0.061, Fig. 3a). We concluded therefore that neither artificial selection nor inbreeding derived from the crossing strategy of our culture system did affect the size of the animals over time.

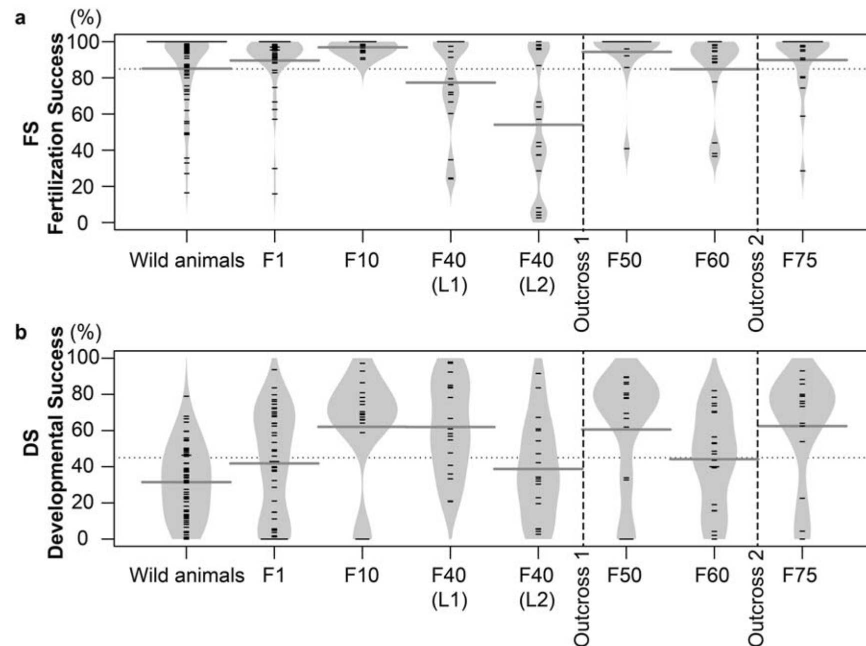

FIG. 3. Analysis of the Fertilization Success (FS) (**a**) and Developmental Success (DS) (**b**) to evaluate the fertility of inbred populations in the *O. dioica* facility. FS and DS values from in vitro fertilization experiments crossing wild animals or inbred animals from the facility (Table 1) are represented as short horizontal lines in one-dimension scatter beanplot (some lines appear bigger because of the coincidence of values in different crosses). Grey areas represent the density distribution of the values, and the thick grey line indicates the average values of FS or DS within each generation. The horizontal dotted line shows the overall FS or DS averages. The FS and DS values of inbred animals for 1 generation (F1), or more than 10 (F10), 40 (F40), 50 (F50), 60 (F60) or 75 (F75) generations were compared with the FS and DS values of wild animals. Interestingly, the low FS and DS averages of wild animals (85% ± 20 and 31% ± 20, respectively) did significantly increase after 10 generations (F10, FS = 97% ± 3 and DS = 62% ± 30; see Table S2 for statistical significance). After maintaining two independent *O. dioica* lines (L1 and L2) for more than 40 generations, however, their FS and DS averages decreased. We then created a new line by crossing animals from L1 with animals from L2 (outcross 1, dashed left line). The FS and DS averages of the new line were improved after the outcross (e.g., F50), but the DS declined again 20 generations later (F60). For this reason, the *O. dioca* population was outcrossed with wild animals at the F74 generation (outcross 2, dashed right line), leading again to a significant improvement of the FS and DS in the next generation F75. The data showed, therefore, that fertility values of the inbred populations in the facility are maintained similar or even better than in wild animals for several generations, but they can decline in the long-term. Periodical outcrosses that increase the genetic variability of the *O. dioica* populations in the facility appear to overcome the decline of fertility because of the inbreeding.

We also counted and photographed the eggs in the spawns of the previously selected females from each group. The averages and standard deviation of clutch sizes from females in F1, F20, and F40 were 283 ± 50; 222 ± 83, and 141 ± 42 eggs, respectively, whereas the average of eggs from nonrelated wild females was 237 ± 81 eggs. The average production of eggs did not show significant differences during at least the first 20 generations (see Table S1 in Supporting Information for *P*-values of the pairwise comparisons). By generation 40, however, the average number was significantly lower, suggesting that the inbreeding or the selection derived from our mating strategy might be diminishing the fecundity of the lines. The outcross of two independent inbred lines stopped, however, this decreasing trend since the hybrid line showed a slight improvement of egg production, which was maintained with no significant differences after 10 or 20 extra generations (164 ± 23 at F50 and 188 ± 44 at F60, respectively). We concluded, therefore, that despite some variability could occur through generations, the overall egg production of our animal facility produced spawns big enough (170 ± 60 eggs in average) for most experimental needs of standard laboratories.

To test if the inbreeding affected the fertility of inbred animals, we measured the percentage of eggs that after fertilization proceeded to the first cleavage (fertilization success, FS) and the percentage of embryos that properly develop at least until hatching (developmental success, DS) through many generations of inbreeding (from F1 to F75). The experimental design for this analysis consisted in making crossing-grids in which a subset of eggs from individual females (e.g., females 1, 2, and 3) were in vitro fertilized with sperm of individual males (e.g., males 1, 2, and 3). The in vitro fertilization protocol followed the next steps. Mature females were individually transferred to glass dishes and rinsed twice with 5 mL of sSW to remove any potential contaminant sperm from the culture. Each female was allowed to naturally release the eggs, and each clutch was subdivided in batches of at least 20 eggs, which were maintained at 13°C for no more than

Table 1
Fertilization Success and Development Success of Wild Animals and Partially Inbred Populations in the O. dioica Facility

	WA	F1	F10	F40 (L1)	F40 (L2)	F50	F60	F75
FS (%±SD)	85 ± 20	90 ± 17	97 ± 3	77 ± 26	54 ± 36	85 ± 22	90 ± 17	94 ± 15
DS (%±SD)	31 ± 20	42 ± 30	62 ± 30	62 ± 25	39 ± 27	61 ± 32	44 ± 26	62 ± 30
Number of eggs	4863	2987	1006	846	848	882	973	613
Number of crosses	65	48	18	18	18	18	21	15

For each generation (from parental wild animals WA to F75 as described in Fig. 3), means (%) and standard deviations (SD) of fertilization success (FS) and development success (DS) were estimated by counting the number of eggs of independent crosses that after in vitro fertilization proceeded to the first cleavage (FS) or properly develop at least until hatch (DS). Wild animals (WA), generations F1-F75, L1 and L2 are different animals lines.

2 hours until being fertilized. Mature males were also rinsed twice in 5 mL of sSW, and placed individually in an eppendorf tube with the smallest possible volume of sSW (e.g., 1–2 μL) to minimize sperm activation after natural spawning but avoiding desiccation. Each male was allowed to naturally release the sperm and just before fertilization, it was diluted in 1 mL of sSW. For the in vitro fertilization, eggs were placed in glass dishes containing 800 μL of sSW and inseminated with 200 μL of the sperm dilution at 19°C. Ten minutes after insemination most polar bodies were visible, eggs were transferred to 3 mL of sSW to avoid polyspermy, and embryo development was allowed to proceed at 19°C. We evaluated the FS and DS values by photographing and counting with ImageJ the number of embryos in each batch that underwent at least one cell division and properly developed until hatching, respectively. Crosses in which one female or one male consistently showed FS below 25% in all its matings were discarded to avoid an underestimation of the FS because of technical problems (i.e., sperm or eggs were not properly manipulated). We performed a total of 156 crosses among inbred animals (Table 1) using 13 females and 11 males from F1, 6 females and 6 males from F10, 16 females and 14 males from F40, 6 females and 6 males from F50, 5 females and 9 males from F60, and 6 females and 6 males from F75. For wild animals, the experiment was done twice performing 65 crosses using 20 females and 19 males from animals collected during the winter-spring seasons of two consecutive years, 2013 and 2014.

The average FS and DS of wild animals were 85% ± 20 and 31% ± 20 (embryos analyzed $n = 4863$; Table 1 and Fig. 3), respectively. In the first generation of inbreeding F1 (embryos analyzed $n = 2987$), the FS was 90% ± 17, and the DS was 42% ± 30, both similar to the FS and DS values of wild animals (Table 1 and Table S2 for *P*-values of pairwise comparisons). Interestingly, the FS and the DS improved after more than 10 generations (F10 embryos $n = 1006$), since FS (97% ± 3) and DS (62% ± 30) were significantly higher than those for wild or F1 animals (P-value < 0.05 and < 0.001, respectively; Table 1 and Table S2). After many generations of inbreeding, however, some of the lines appeared to suffer a reduction of their FS and/or DS average values (e.g., see for example FS and DS in the F40 of line 2 or DS in F60 in Fig. 3). To overcome this limitation, we periodically followed an outcrossing strategy either by "internally outcrossing" inbred lines that had been independently maintained (outcross 1 in Fig. 3), or by "externally outcrossing" inbred lines with newly collected wild animals (outcross 2 in Fig. 3) to reduce the inbreeding of the *O. dioica* populations in the facility. These outcrosses resulted in significant improvements of the FS and DS average values as can be observed for instance in the FS average value after outcross 1 (F50) and 2 (F75), or the DS average value after outcross 2 (F75) (P-values < 0.05).

Overall, our data indicated that our animal facility is capable to maintain *O. dioica* populations in a healthy reproductive status through many generations, showing FS and DS values similar or even higher than those from wild animals. Despite that this status is not stably maintained since egg production, and the FS and DS parameters might decline after many generations of inbreeding, we have shown that a periodical outcrossing strategy appears as a possible solution to overcome this limitation.

DISCUSSION

Optimization of the *O. dioica* Facility to Develop a Low-Cost System

For many decades, the husbandry of *O. dioica* has been challenging for two main reasons. First, the difficulty to maintain this fragile planktonic animal that lives within a house suspended in a frequently refreshed seawater system. Second, fulfilling the feeding needs for maintaining the physiology and fecundity of *O. dioica* in healthy conditions. Recently, two main facilities—in the SARS Institute and in the University of Osaka—have successfully addressed these challenges and have been able to construct a husbandry system capable to indefinitely maintain *O. dioica* populations throughout the year (Bouquet *et al.*, 2009; Nishida, 2008). The SARS facility, for instance, routinely maintains four or five populations, and can generate 6,000 mature animals

per week, which allows the capability to produce tens of thousands of embryos any given day (Bouquet *et al.*, 2009). The production of *O. dioica* at this large scale, however, requires significant amount of space, specialized equipment, large volumes of seawater, and extensive human resources (Bouquet *et al.*, 2009). Our aim was to investigate how to scale-down previously described *O. dioica* culture systems in order to minimize the amount of space, human resources, and technical equipment, but affecting the less as possible to animal production. In comparison to large-scale *O. dioica* production systems, the simplification implemented in our husbandry system implies 95% savings of seawater (from 220 liter to 12 liter per generation), 93% savings of beaker manipulation (from 29 to 2 beakers per generation cycle), 90% savings of beaker maximum occupation (from 10 to 1 beaker in any given day per line), and 87% savings of alga-production (from 2 liter to <0.25 liter per generation). We have also considerably minimized labor tasks such as a 94% reduction in the number of animals to be transferred per line (from more than 2400 to 150), and a reduction to half in the feeding frequency (once rather than twice per day). In our case, the total time dedicated by one person to the maintenance of each animal line is ~3 hours per cycle, ranging from 20 minutes in days D1, D2, and D4, to 1 hour on D0 and D3. In the case of microalgal culturing, we spend 1 hour per week for the setting up of the feeding cultures, and 1 hour per month for long-term stock renewal. Obviously, these savings and reductions were accompanied by a significant cutback of the production capability of our facility (see Discussion below), and the trade-off between production and cost of the facility is a parameter that should be considered in the face of the requirements and resources of each laboratory.

Other important aspects we have also improved are the tasks of maintenance of microalga stocks. The use of long-term liquid or solid cultures of microalgae allowed us to renew the stocks only once every month (Supporting Information Fig. 1), and to isolate single colonies from solid cultures in case of accidental contamination (Fig 1b). We have also developed novel protocols to cryopreserve the four species of microalgae, which allowed us to store microalgae stocks in liquid nitrogen without any additional manipulation, and to recover microalgal cultures in case of accidental loss of a strain or contamination.

Production of a "Low-Cost" *O. dioica* Facility

Our *O. dioica* facility has been able to maintain multiple inbred lines during 2 years, some of them over 75 generations with sporadic outcrosses between genetically nonrelated lines or wild animals. We normally maintain at least two independent inbred lines, and a set of three nonsynchronized "backup" cultures with animals that spontaneously breed. Typically, our *O. dioica* facility generates more than 150 mature animals per week, which allows us to produce more than 10,000 embryos. Despite our production is far below the 6000 mature animals reported for the SARS Institute facility, our system is expandable on demand easily 10-fold by increasing the number of matings at day 0, and by increasing the number of animals transferred at day 3 (Fig. 1j).

The characterization of the animals of our facility after many generations of inbreeding reveals that the average length tail (2.1 mm), which is a measure of animal size that positively correlates with trunk length, gonad length and house size (Lobón *et al.*, 2011), is not significantly different from wild animals collected in the coast of Barcelona. We can conclude, therefore, that the size of the animals did not seem to be affected by the environmental conditions of our husbandry system, neither our mating strategy nor the inbreeding and selection accumulated in our laboratory lines. This average length, however, is lower than the average length reported in animals collected in other locations [e.g., 3.3 mm in animals from the Cantabric sea in the north coast of Spain, (Lobón *et al.*, 2011)], which suggests that length may vary among different populations.

Despite the size of the animals of our facility did not seem to significantly change through generations, we have observed that parameters related to fecundity such as egg production, or fertility such as fertilization and developmental success did appear to be susceptible to the inbreeding accumulated by our mating strategy. For this reason, we suggest to periodically test for egg production, fertilization success, and developmental success, for instance every 20 generations, to evaluate the status of the animal populations of the facility. We also recommend the maintenance of some genetic variability in the facility such as independent inbred lines or independent "backup" beakers, or the sporadic input of new genetic variability from wild animals or cryopreserved sperm (Ouchi *et al.*, 2011) to make out-crosses in order to reduce inbreeding effects when detected. Similar outcross strategies have been also recommended for large-scale facilities (Bouquet *et al.*, 2009), in which values of developmental success around 80–90% are typically observed. The application of out-cross strategies to compensate the negative effects of inbreeding appears therefore fundamental to maintain the cultures healthy, specially in the case of small-scale *O. dioica* facilities like ours that hold a limited amount of genetic variability. The overall average egg production in our facility was 170 ± 60 eggs per female, which is lower than in wild animals collected in the coast of Barcelona (237 eggs per female), or animals from the SARS institute in Norway (303–388, Bouquet *et al.*, 2009; Troedsson *et al.*, 2002), but similar to the reported 165 eggs per female of animals

from the SARS raised in Villefranche-sur-Mer (France) (Lombard *et al.*, 2009), or higher than the 122 eggs per female from animals from the Cantabric sea (Lobón *et al.*, 2011). It is clear, therefore, that in addition of the differences in egg production that may exist among populations, parameters such as feeding frequency, animal density, and seawater renewal of the animal culture can significantly affect egg production. Overall, these parameters have been optimized in large-scale facilities for producing large quantities of biological material, while in our case we prioritized the reduction of the costs of the facility. Our husbandry protocol opens the possibility that each laboratory can choose the production/cost trade-off that best agrees with its requirements and resources.

We found interesting that the average value of the developmental success was unexpectedly low in wild animals (31% ± 20), and that this value significantly improved after 10 generations (62% ± 30). A possible explanation is that our breeding system artificially selects those variants that better adapt to our laboratory conditions, which will be already widespread after 10 generations. An alternative—although not mutually exclusive—explanation is that the low value of the developmental success in wild animals is due to the mutational load resulting from accumulated deleterious mutations in wild populations. The mutational load in *O. dioica* could be high because of the significant population mutation rate reported for this species, $\theta = 4Ne\mu = 0.0220$ (Denoeud et al., 2010). The combination of the purifying selection and the genetic bottlenecks of our mating strategy may be causing the purge of many deleterious mutations, resulting in improved FS and DS values during the first generations. This purge, however, could be accompanied by a slow widespread of some recessive lethal mutations by genetic drift that started to have a negative effect on FS and DS values due to the inbreeding generated after many generations, for example, F40. This negative effect of the inbreeding on the fertility parameters would be compatible with the observation that fertility can be improved by outcrossing independent inbred lines or by occasionally introducing wild animals in the system, which entail an increase on the genetic variability of the *O. dioica* populations in the facility. Further analyses of *O. dioica* genetic variability related to developmental genes in natural populations will help to find out the mechanisms responsible for the low rate of developmental success in wild animals and to better understand the developmental constraints and plasticity that underlay the evolution of the mechanisms of development, a central question of Evo-Devo.

CONCLUSIONS

In conclusion, we have described how to set up an *O. dioica* "low-cost" facility that is reliable, flexible, easily expandable on demand, and capable to produce thousands of embryos and hundreds of adults every week. Our results suggest that, eventually, inbreeding may affect the fertility of the laboratory populations over many generations, a limitation that can be overcome with outcrossing strategies. Our optimization of the husbandry system of *O. dioica* also includes new protocols for microalgae long-term maintenance and cryopreservation. We hope that the facility described here will facilitate the research with *Oikopleura* and pave the way for spreading *O. dioica* as a model organism.

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