

Complex heterochrony underlies the evolution of *Caenorhabditis elegans* hermaphrodite sex allocation

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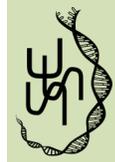
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Hermaphroditic organisms are key models in sex allocation research, yet the developmental processes by which hermaphrodite sex allocation can evolve remain largely unknown. Here we use experimental evolution of hermaphrodite-male (androdioecious) *Caenorhabditis elegans* populations to quantify the developmental changes underlying adaptive shifts in hermaphrodite sex allocation. We show that the experimental evolution of increased early-life self-fertility occurred through modification of a suite of developmental traits: increased self-sperm production, accelerated oogenesis and ovulation, and increased embryo retention. The experimental evolution of increased self-sperm production delayed entry into oogenesis—as expected, given the sequentially coupled production of self-spermatogenesis and oogenesis. Surprisingly, however, delayed oogenesis onset did not delay reproductive maturity, nor did it trade-off with gamete or embryo size. Comparing developmental time dynamics of germline and soma indicates that the evolution of increased sperm production did not delay reproductive maturity due to a globally accelerated larval development during the period of self-spermatogenesis. Overall, heterochrony in gametogenesis and soma can explain adaptive shifts in hermaphrodite sex allocation.

KEY WORDS: *Caenorhabditis elegans*, evolution of development, experimental evolution, hermaphrodite, mating system evolution.

Understanding how organisms allocate their resources to different sexual functions is important to understand life-history evolution. In many taxa, sex allocation may be realized through the maintenance of separate sexes, each specialized in female versus male reproductive functions. Alternatively, species may show co-sexuality, that is, hermaphroditism where individuals perform both female and male reproductive functions. Self-fertilization (selfing) through hermaphrodites is common in animals and plants (Stebbins 1957; Jarne and Auld 2006; Weeks et al. 2006; Barrett 2008), and a central question is how natural and sexual selection operates on the allocation to male versus female function within individuals of hermaphroditic organisms.

Fisher's insight that negative frequency-dependent selection determines the evolution of sex ratios (Fisher 1930) can be used to understand how selection operates on hermaphrodite sex allocation. Specifically, the marginal fitness returns of alleles responsible for conflicting sexual functions in hermaphrodites are expected to be minimized during evolution (Charlesworth and Charlesworth 1981; Charnov 1982; Lloyd 1984). It is generally understood that if hermaphrodites have simultaneous sex functions, availability of mates and breeding seasonality may determine how natural and sexual selection will favor changes on developmental time to maturity (Schärer 2009; Escobar et al. 2011). When hermaphrodites predominantly self, the even



allocation of resources toward spermatogenesis and oogenesis should be favored (Charnov 1987). As reproduction is usually oocyte-limited for most organisms, female-biased sex allocation is expected to be favored under predominant selfing (Weeks 2012).

Some of these ideas have been experimentally examined by manipulating mating or cross-pollination opportunities (Tian-Bi et al. 2008; Dorken and Pannell 2009; Roels and Kelly 2011), the level of resources available for reproduction and survival (Mazer et al. 2003; Case and Ashman 2007), and by manipulating the reproductive system (Teotonio et al. 2012; Carvalho et al. 2014b; Theologidis et al. 2014). Despite these studies, however, it remains mostly unknown which developmental changes underlie the evolution of hermaphrodite sex allocation under selfing and/or outcrossing (Li and Johnston 2010; Roels and Kelly 2011; Noel et al. 2016). In general, it is evident that the evolution of hermaphrodite sex allocation may occur through selection on the timing and rates of gametogenesis and other developmental processes associated with sexual functions (Alberch et al. 1979; Lord and Hill 1987; Li and Johnston 2000; Mazer et al. 2004). Such “heterochrony”—defined here as an evolutionary change in the relative timing or relative rates of developmental processes (Alberch et al. 1979; Raff 1996; Li and Johnston 2000)—has been suggested to underlie the evolution of selfing rates and transitions between selfing and outcrossing (Li and Johnston 2000).

Evolutionary transitions to selfing have been correlated with the evolution of developmental time to maturity (Lord and Hill 1987; Weeks et al. 2006; Tian-Bi et al. 2008; Escobar et al. 2011) and, in the case of angiosperms, with differential timings and rates of germline and somatic tissue development within flowers (Darwin 1877; Harder and Barrett 2006; Li and Johnston 2010; Geuten and Coenen 2013). Most of these studies have inferred heterochrony based on comparative analysis, and we therefore lack direct measurements of developmental processes in well-defined evolutionary contexts. Here we use experimental evolution with *Caenorhabditis elegans* to measure heterochrony in germline and somatic development underlying sex allocation, heterochrony that we previously suggested may explain the adaptive evolution of self-fertility (Carvalho et al. 2014b).

The nematode *C. elegans* exhibits androdioecy, a reproductive system consisting of self-fertile hermaphrodites and males (Maupas 1900). Within the *Caenorhabditis* genus, androdioecy derived from male–female dioecy at least three times independently through distinct changes in germline sex determination (Braendle and Félix 2006; Kiontke et al. 2011). In natural populations, *C. elegans* hermaphrodites primarily self and males are rarely found (Barriere and Félix 2005; Félix and Braendle 2010). Consistent with a long history of predominant selfing,

inbreeding depression is low (Dolgin et al. 2007; Gimond et al. 2013); moreover, sexual selection should have been of minor consequences and, as expected, hermaphrodites appear to have lost the ancestral female ability to produce mating pheromones and are reluctant to mating with males (Lipton et al. 2004; Chasnov et al. 2007). Unlike most hermaphroditic animals and plants, *C. elegans* hermaphrodites cannot mate with each other and thus exclusively function as females when outcrossing occurs through male mating. The “male” reproductive function of *C. elegans* hermaphrodites is thus limited to self-spermatogenesis, self-sperm activation, and self-sperm fertilizing capacity, and represents an exclusive component of selfing, being better termed “selfing” reproductive function. In contrast, female reproductive functions of *C. elegans* hermaphrodites encompass oogenesis, ovulation, mating and egg laying, and are common to both selfing and outcrossing. The inability of hermaphrodites to fertilize each other renders *C. elegans* particularly useful as selfing rates can be unambiguously determined as one minus twice the male frequency of the population (Stewart and Phillips 2002).

Caenorhabditis elegans hermaphrodites are protandrous, with early germ cells differentiating into self-sperm, followed by an irreversible switch to oogenesis around the larval–adult moult transition (Hirsh et al. 1976). Due to this sequential nature of gametogenesis, potentially allowing oogenesis to continue until death, selfing hermaphrodites are self-sperm limited under most demographic circumstances (Barker 1992; Cutter 2004). Importantly, due to the sequential coupling of spermatogenesis and oogenesis, increasing self-sperm production (by means of a prolonged period of self-spermatogenesis) causes a delay in the onset of oogenesis and reproductive maturity, thus generating a fitness trade-off between generation time and lifetime fecundity (Hodgkin and Barnes 1991; Cutter 2004). The timing of the switch from self-spermatogenesis to oogenesis represents a direct regulatory mechanism of hermaphrodite sex allocation, so that timing shifts of this switch represents an obvious candidate mechanism underlying the evolution of sex allocation in *C. elegans* hermaphrodites.

While outcrossing is rare in natural *C. elegans* populations, intermediate frequencies can be maintained when populations with standing genetic variation for male outcrossing ability evolve in experimental environments (Anderson et al. 2010; Teotonio et al. 2012; Carvalho et al. 2014b; Manoel et al., 2007; Masri et al., 2013; Morran et al., 2009). In the experiments of Teotonio et al. (2012), partial selfing was stably maintained at 50% over 100 generations of adaptation to a laboratory environment characterized by discrete and nonoverlapping 96-h life cycles at constant and high population sizes ($N = 10^4$). The maintenance of partial selfing in these populations can be explained by a counterbalance in evolutionary improvements of male outcrossing performance

versus hermaphrodite selfing performance (Carvalho et al. 2014ab). The evolution of an increase of hermaphrodite self-reproduction at earlier ages relative to total reproduction is consistent with adaptation to the imposed shortened life cycle during experimental evolution, and may reflect a response that promotes selfing and/or limits outcrossing (LaMunyon and Ward 1998; Carvalho et al. 2014b). Interestingly, evolved hermaphrodites did not show increased reproduction at an early age when fertilized by (tester) males (LaMunyon and Ward 1998; Carvalho et al. 2014b). Because the female function in *C. elegans* hermaphrodites is common to both selfing and outcrossing, this result suggests that hermaphrodite self-spermatogenesis and self-sperm production—as the developmental component exclusive to selfing—must have been increased or accelerated during evolution (LaMunyon and Ward 1998; Carvalho et al. 2014b). Consequently, because increased self-sperm production delays reproductive maturity of selfing *C. elegans* hermaphrodites (Hodgkin and Barnes 1991; Cutter 2004), the observation that evolved hermaphrodites also showed increased early-life self-fertility appears paradoxical: how can evolution of increased hermaphrodite self-sperm production be developmentally accommodated without causing a delay in reproduction, that is, decreased early-life self-fertility? Diverse evolutionary changes in development, including heterochrony, could explain this seemingly contradictory finding; however, the specific changes in hermaphrodite sex allocation and developmental timing that occurred during these evolution experiments remain to be determined.

The above experimental results motivated the present study to measure evolutionary changes in hermaphrodite development. We therefore specifically test the hypothesis that the evolution of increased self-fertility can be explained by changes in hermaphrodite sex allocation. Using the same *C. elegans* experimental populations (Carvalho et al. 2014a,b; Fig. 1A), we quantified in parallel the relative differences in developmental and reproductive traits between selfing hermaphrodites of ancestral (generation 0) versus evolved populations (generation 100), in the context of the 96-h life cycle imposed during experimental evolution. We followed the dynamics of hermaphrodite germline and somatic development throughout the life cycle to quantify somatic developmental timing, germline proliferation and differentiation, transition from spermatogenesis to oogenesis, ovulation, and gamete size (Fig. 1B, C). In parallel, these developmental traits were integrated with measurements of relevant reproductive output traits, including the timing of reproductive maturity, offspring number and size, and reproductive schedules. Using these measures, we ask whether heterochrony in germline and/or soma was in part responsible for the evolution of increased hermaphrodite self-fertility, and as a consequence, whether such sex allocation heterochrony can explain the adaptive maintenance of partial selfing.

Materials and Methods

EXPERIMENTAL EVOLUTION LIFE CYCLE AND EXPERIMENTAL DESIGN

All results reported here are based on the experimental evolution of androdioecious *C. elegans* populations in the context of the imposed 96-h life cycle (Fig. 1A). The ancestral population (G0) was constructed by the successive pairwise intercrossing of 16 isogenic wild isolates. Starting from this population, several independent replicate populations were cultured for 100 generations at 20°C and 80% relative humidity under a discrete time, 96-h nonoverlapping life cycle with a population census size of 10^4 individuals (from first larval stage to adulthood), and effective population sizes of about 10^3 (Teotonio et al. 2012; Chelo and Teotonio 2013). Three of these G100 replicate populations (labeled A4, A5, and A6) were examined in the present study. After 100 generations, populations continued to be genetically diverse, with average genome-wide heterozygosity of about 0.3 (Chelo and Teotonio 2013). At 24 h of the life cycle, 10^4 L1-staged worms were seeded in 10 Petri plates with NGM-lite agar (U.S. Biological) and covered with an *Escherichia coli* HT115 lawn that served as ad libitum food until reproduction. After 72 ± 2 h, corresponding to 96/0 h (end/starting point) of the life cycle, adults from all plates were mixed and killed using a hypochlorite “bleach” solution and embryos harvested in an M9 solution (Stiernagle 1999). After 24 ± 2 h, hatched embryos became starvation-arrested L1s, upon which appropriate density estimation is seeded into fresh plates to constitute the following generation (Fig. 1A). Most evolutionary trait changes are expected to be due to the sorting and recombination of standing genetic variation (Teotonio et al. 2009; Denver et al. 2010). Further information on the experimental evolution design is presented in Figure 1A and in Teotonio et al. (2012).

LIFE HISTORY AND DEVELOPMENTAL ASSAYS

For all the assays reported here, the ancestral (G0) and the three evolved (G100) populations were thawed from frozen -80°C samples (each with $N > 10^3$ individuals) and passaged in parallel for two generations under the standard conditions of experimental evolution. This allowed us to concurrently assay ancestral and evolved populations, control for transgenerational environmental effects and account for potential lab biases.

To confirm the adaptive significance of partial selfing, we determined reproductive schedules of individual selfed hermaphrodites as in Carvalho et al. (2014b). Fifty-six-hour L4 larvae were sampled and transferred to fresh plates every 24 h until offspring production ceased. The number of hatched larvae (L1–L3 stages) was scored, for an average of 82.5 ± 5 SD individuals per population sample. At 96 h of the life cycle, embryo number in utero was quantified in randomly picked hermaphrodites

(Fig. 1C), for 100 observations per population. Embryo size was quantified at 96 h for 200 embryos per population.

Hermaphrodite spermatid area was scored as a proxy for sperm size of dissected young adults (LaMunyon and Ward 1999) that had been isolated 24 h before the L4 stage to prevent mating with males. An average of 12.5 ± 8.0 SD sperm in each of 28–40 individuals were measured per population. Sperm number in young adult hermaphrodites that had been isolated at the L4 stage

was measured for an average of 76.3 ± 3.3 SD individuals per population.

To characterize spermatogenesis and the timing of the sperm–oocyte switch, at the mid-L4 stage, we examined the proportion of individuals entering spermatogenesis, as inferred from the presence of primary/secondary spermatocytes and mature sperm (an average of 62.5 ± 4.2 SD individuals per population was measured). This was done using whole-body

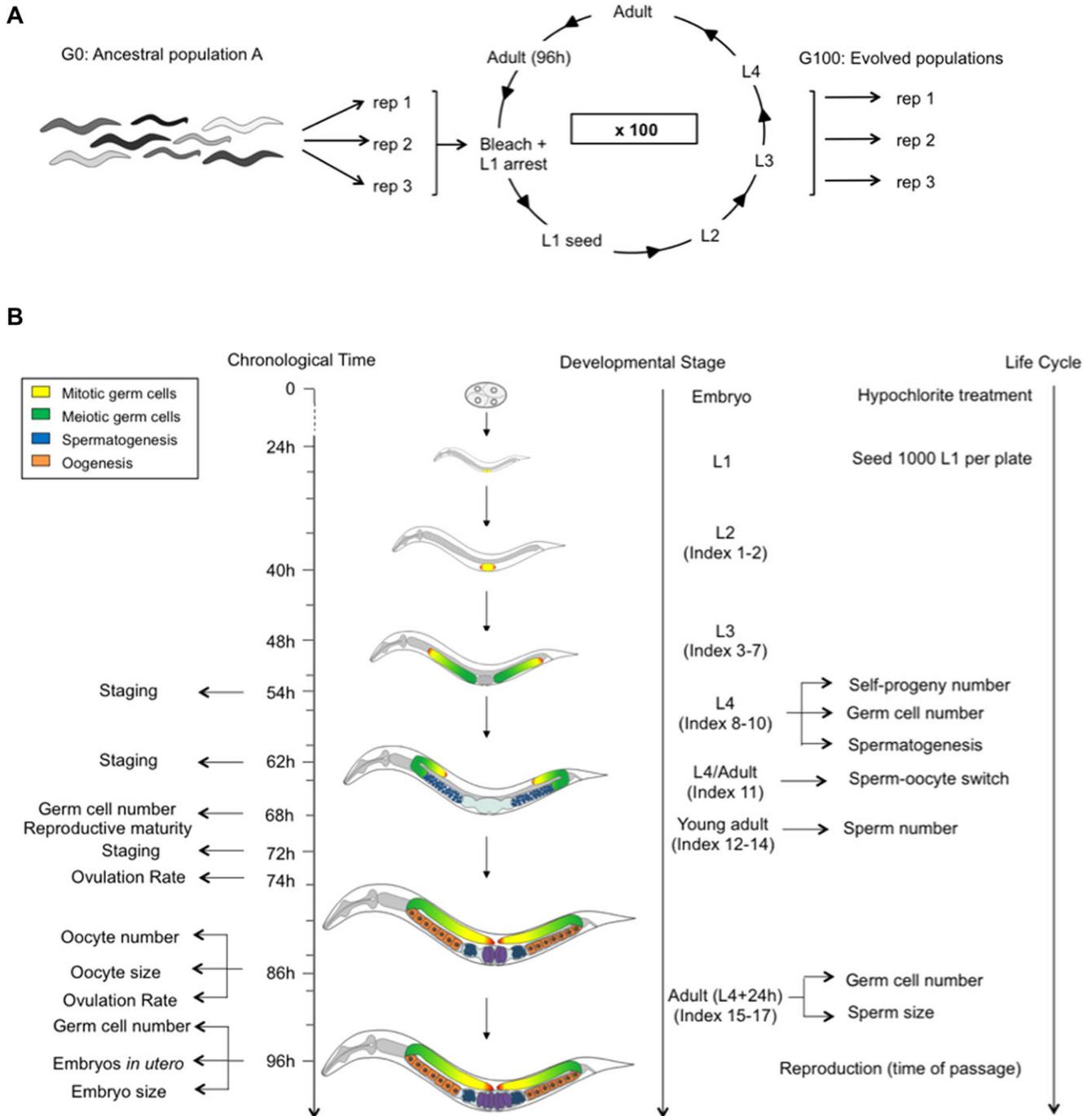


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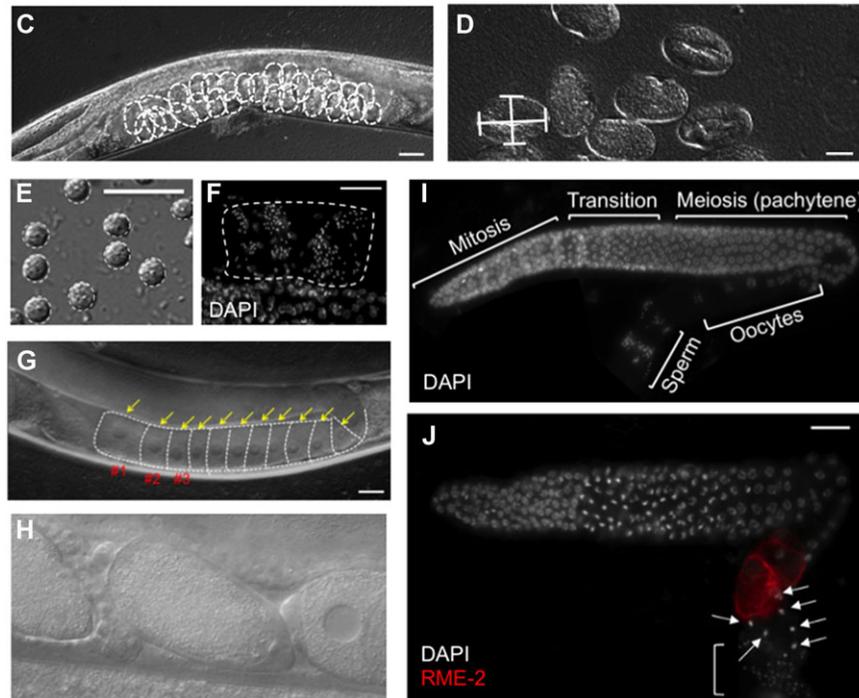


Figure 1. Life cycle during experimental evolution, hermaphrodite germline development, and experimental design. (A) Outline of the experimental evolution assay and 96-h life cycle (Teotonio et al. 2012). The ancestral androdioecious population G0 was derived from a funnel pairwise cross of 16 *C. elegans* wild isolates. Starting from this population, several independent replicate populations were cultured for 100 generations at 20°C following the same 96-h life cycle (Fig. 1A, Teotonio et al. 2012), and three of these replicate populations (G100, rep. 1–3) were examined in this study. The time of passage between generations is defined as the life cycle end time point of adults at 96 h, which are harvested to collect embryos, defining the starting time point of the life cycle (0 h) of the subsequent generation. Collected embryos were maintained in liquid medium without food for 24 h during which they hatch into L1 larvae that arrest development. At this time point (24 h), age-synchronized L1 cohorts were seeded onto agar plates with bacterial food at a constant population size ($N = 1000/\text{plate}$). Populations were then allowed to develop and reproduce for 72 h until the time of passage (96 h) at which time individuals were washed off plates, treated with a bleach-hypochlorite solution to harvest embryos, initiating the next generation. (B) Timeline of phenotyping assays performed in this study. All phenotypes were measured in the same life cycle as imposed during the 100 generations of experimental evolution (Teotonio et al. 2012). Phenotypes were concurrently scored in the ancestral population and three evolved replication populations. All phenotypes—except ovulation rates—were measured in parallel in a single experiment (see Supporting Information). Different phenotypes were quantified in chronologically staged hermaphrodites at different time points (left) and in hermaphrodites of morphologically defined, distinct developmental stages (right). The drawings illustrate the developmental progression of the hermaphrodite through the four larval (L1–L4) and adult stages during the life cycle. Timing of developmental events is based on observations of the ancestral population. Different colors depict mitosis–meiosis progression and sperm–oocyte allocation in the developing hermaphrodite germline, based on published information (Kimble and Ward 1988; Kimble and Crittenden 2007). Distal tip cells are indicated in red. (C–J) Illustration of a subset of scored phenotypes (based on individuals from the ancestral population). (C) DIC (Differential Interference Contrast) microscopy image of an adult hermaphrodite at 96 h of the life cycle. Embryos in utero are outlined in white. (D) DIC image of embryos collected at 96 h of the life cycle to estimate embryo size. (E) DIC image of dissected spermatids of an early adult hermaphrodite, used for estimating sperm size. (F) Microscopy image of a DAPI-stained hermaphrodite at the early adult stage, outlining spermatids in spermatheca and uterus. (G) DIC image of the posterior gonad arm in an adult hermaphrodite at 86 h of the life cycle. Enlarged oocytes developing after the germline loop region are indicated with yellow arrows. The three most proximal oocytes, labeled 1–3, were used to estimate oocyte size. (H) Nomarski images of spermatheca (with uterus on left, proximal germline on right) in an ovulating hermaphrodite (74 h of life cycle). (I) Microscopy image of an extruded DAPI-stained gonad arm of an adult hermaphrodite (distal–proximal axis oriented from left to right). Mitotic and meiotic (pachytene) nuclei can be distinguished using nuclear morphology. The transition zone delineates the entry into meiosis, apparent by crescent-shaped nuclei (Francis et al. 1995; Crittenden and Kimble 2008). (J) Microscopy image of dissected gonad arm of a hermaphrodite at the L4/adult stage (ancestral population), stained with DAPI (white) and RME-2 antibody (red). This protocol was used to infer measures on entry into spermatogenesis and oogenesis, respectively. Mature spermatids are located in the most proximal zone of the gonad, delineated by the white bracket; distinct large nuclei of primary spermatocytes are indicated by arrows. Staining with RME-2 indicates presence of differentiating oocytes in the proximal germline.

DAPI (4',6-diamidino-2-phenylindole) stains. At the L4/adult transition (moult), we scored each individual according to the index: (1) presence of mature spermatids; (2) presence of spermatids and expression of an early oogenesis marker, RME-2 (Grant and Hirsh 1999); and (3) presence of spermatids, RME-2 expression, and presence of enlarged diakinesis-stage oocytes. An average of 41 ± 3.5 SD individuals per population were measured. Because of the antibody staining, these observations were made using dissected DAPI-stained gonad arms.

We estimated the proportion of ovulating individuals of randomly selected hermaphrodites by scoring the presence or absence of oocytes in the spermatheca (Fig. 1H). A total of 255 individuals (at 74 h) and 355 individuals (at 86 h) were examined per population. At 86 h, for an average of 45.8 ± 0.9 SD individuals per population, we counted the number of enlarged oocytes at diplotene and diakinesis of Prophase I, developing in the loop region (McCarter et al. 1999, Fig. 1G), and we measured the area of the three most proximal oocytes adjacent to the spermatheca (McCarter et al. 1999, Fig. 1G). We quantified the number of germ cell precursors in hermaphrodites in two somatic stages, mid-L4 and L4 to adult moult, and two chronological stages: 68 and 96 h. For all individuals, a single gonad arm was extruded and DAPI-stained to count total germ cell number and to distinguish mitotic versus meiotic germ cell fate, following previously described protocols (Poullet et al. 2015, Fig. 1I). For each of the four examined developmental stages, analysis included on average 43.4 ± 9.0 SD individuals per population.

At 54, 62, and 72 h of the life cycle, an average of 192 ± 24 SD randomly selected hermaphrodites per population was scored for their developmental stage, according to Table S1. Stages of indices 1–11 were based on somatic markers and stages of indices 12–17 on reproductive markers. For analysis, we first calculated the frequency-weighted average of the numerical index. Detailed assay descriptions can be found in Supporting Information materials and methods and Figure 1.

STATISTICAL ANALYSIS

Data was analyzed with linear mixed effects models (LMM) or generalized linear mixed effect models (GLMM) and REML estimation methods (Pinheiro and Bates 2000), to test for significant evolutionary responses, by taking generation as a fixed two-level factor and individual (if needed be) nested within population nested within block as random factors. Taking population as a random factor irrespective of generation accounts for the effects of genetic drift and other historical accidents during evolution, while being conservative as the ancestral population variation is considered. Progeny number until 96 h over lifetime progeny, and counts of individuals that had begun spermatogenesis versus those that had not, were analyzed with GLMM assuming binomial errors. Embryo number in utero and number of oocytes were ana-

lyzed with GLMM assuming Poisson errors. All other traits were modeled with LMM. Model residuals were tested for normality with Shapiro–Wilk tests, when evolutionary responses were significant. In the cases of spermatogenesis entry, sperm–oocyte transition and reproductive maturity in staged hermaphrodites, outlier removal (residuals >2.5), and data transformation were unsuccessful in normalizing residuals. In these cases, LMM or GLMM models were performed without considering generation as a factor, and likelihood ratio tests (LR test) done to test whether inclusion of generation improved the models' fit. LR test significance was determined assuming a chi-squared distribution with one degree of freedom, and also by parametric bootstrapping (PB test; Halekoh and Højsgaard 2014). For all other traits, planned contrasts among generations were done with Student *t*-tests with the effective number of degrees of freedom being calculated with the Kenward–Roger approximation (Halekoh and Højsgaard 2014; Lenth 2014). Even when evolutionary responses were not significant, we present in all plots the least-square estimates from the models. The packages *stats*, *lme4*, *lsmeans*, and *pkbrtest* in R were used for computation (R Development Core Team 2013).

Results

EVOLUTION OF HERMAPHRODITE SELF-FERTILITY AND REPRODUCTIVE SCHEDULE

We first compared reproductive schedules of selfing hermaphrodites between ancestral and evolved hermaphrodites to confirm the adaptive significance of partial selfing during experimental evolution (Carvalho et al. 2014b). Consistent with adaptation to the experimental 96-h life cycle, evolved hermaphrodites produced a higher number of selfed offspring until 96 h (time of passage) when concurrently compared to ancestral hermaphrodites (Fig. 2A, one-tailed $t_{5,4} P = 0.03$), while lifetime number of offspring was unchanged (Fig. 2B, $t_{5,1} P = 0.67$). There was thus a shift toward increased early self-reproduction with experimental evolution as expected with adaptation (Williams 1957; Anderson et al. 2011). We further found that at 96 h evolved hermaphrodites exhibited a much higher number of embryos in utero (Fig. 2C, $t_{4,2} P < 0.01$), indicative of strong embryo retention. In contrast, embryo size did not change during evolution (Fig. 2D, $t_5 P = 0.4$). Together, these findings suggest that the evolution of improved hermaphrodite reproduction was partly due to the evolution of increased embryo retention, probably as a direct response to the hypochlorite treatment imposed at 96 h of the life cycle, throughout experimental evolution and to which only embryos survive (Stiernagle 1999; Teotonio et al. 2012).

EVOLUTION OF SPERMATOGENESIS

Hermaphrodites of evolved populations produced significantly more sperm than hermaphrodites of ancestral populations

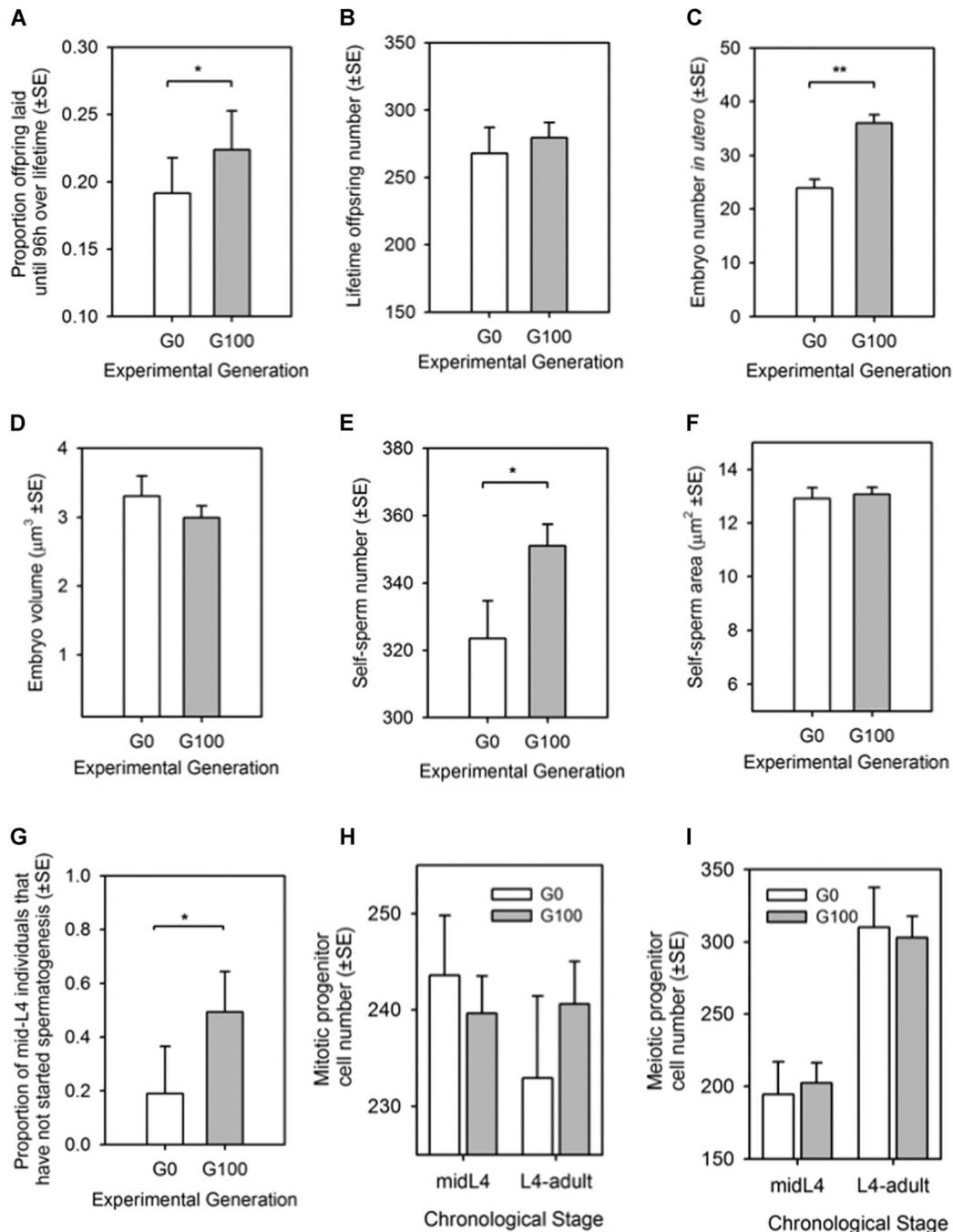


Figure 2. Evolution of reproduction, embryonic traits, sperm traits, and germ cell progenitors. (A) Reproductive schedules of self-fertilizing hermaphrodites in ancestral and evolved populations. Evolved population showed a significant shift toward earlier reproduction. (B) Lack of experimental evolution responses in lifetime offspring production of selfed hermaphrodites. (C) Number of embryos in utero at 96 h, the time of passage during experimental evolution. There was an increase of embryo number in utero during experimental evolution. (D) Embryo size (volume, $3 \times 10^{-4} \mu\text{m}^3$) measured at 96 h did not differ between ancestral and evolved populations. (E) Hermaphrodite self-sperm number significantly increased with experimental evolution. (F) Hermaphrodite self-sperm size (cross-sectional area, μm^2) did not significantly differ between evolved and ancestral populations. (G) Evolved populations showed a significantly lower proportion of mid-L4 individuals that had initiated sperm differentiation (formation of primary spermatocytes). (H) Mitotic germ cell numbers (per gonad arm) for somatically staged individuals (mid-L4 and L4/adult moult). (I) Meiotic (pachytene) germ cell numbers (per gonad arm) for somatically staged individuals (mid-L4 and L4/adult moult). Linear mixed effects and generalized linear mixed effects models used to test for differences among generation 0 (G0) and generation 100 (G100): * P -values < 0.05, ** P -values < 0.01, *** P -values < 0.001. Means and error least-square estimates are shown. See main text for summary statistics.

(Fig. 2E, one-tailed $t_5 P = 0.04$); in contrast, hermaphrodite sperm size did not evolve (Fig. 2F, $t_{4,3} P = 0.75$). In somatically staged mid-L4 larvae, we further found that the germline of all individuals in the ancestral population contained primary spermatocytes and/or mature spermatids while a significant proportion of individuals in evolved populations had not yet started spermatogenesis (Fig. 2G; one-tailed $t_{4,1} P = 0.03$, LR test $P = 0.02$, PB test $P = 0.05$). Evolution of increased self-sperm production was thus accompanied by a delayed entry into spermatogenesis relative to somatic developmental stage. This result suggests that evolution of increased sperm production caused a delayed entry into spermatogenesis, so that the increased allocation of precursors to the sperm fate delays the onset of spermatocyte formation. However, the number of germ cell progenitors in mid-L4 larvae, when spermatogenesis is just beginning did not differ between evolved and ancestral hermaphrodites (Fig. 2H, I; mitotic: $t_{61} P = 0.57$, meiotic: $t_{15,3} P = 0.77$). Similarly, at the L4-adult moult, the numbers of precursor cells in the mitotic or in the meiotic phases did not differ between evolved and ancestral hermaphrodites (Fig. 2H, I; mitotic: $t_{144} P = 0.6$, meiotic: $t_{30} P = 0.82$).

EVOLUTION OF THE SPERM-OOCYTE SWITCH

Without the need to invoke changes in the rates of spermatogenesis, the evolution of increased hermaphrodite sperm production (and delayed entry into spermatogenesis) could have resulted in a delay of the transition between spermatogenesis and oogenesis as they are sequentially connected (Kimble and Ward 1988; Kimble and Crittenden 2007). To confirm this scenario, we inferred the timing of the sperm–oocyte switch using an index that takes into account three distinct events of gamete differentiation and maturation in somatically staged hermaphrodites at the L4/adult moult: presence of mature spermatids, expression of the early-oogenesis marker RME-2 (Grant and Hirsh 1999), and presence of oocytes (diplotene–diakinesis stages) in the proximal gonad arm. As expected, we found a decrease in the index and thus what can be interpreted as a small but significant delay in the sperm–oocyte switch and delayed entry into oogenesis, relative to soma (Fig. 3A; one-tailed $t_{4,1} P = 0.03$, LR test $P = 0.02$, PB test $P = 0.05$). This result is thus consistent with the observations of delayed entry into spermatogenesis and prolonged spermatogenesis in evolved hermaphrodites.

EVOLUTION OF OOGENESIS AND OVULATION

Contrary to expectations (Hodgkin and Barnes 1991; Murray and Cutter 2011), the observed delay in the switch between spermatogenesis and oogenesis did not result in a delay in reaching reproductive maturity since the proportion of individuals with oocytes and first embryos was comparable between evolved and ancestral populations at 68 h of the life cycle (Fig. 3B; one-tailed $t_{5,6} P = 0.17$, LR test $P = 0.29$, PB test $P = 0.27$).

The observations of much higher proportions of individuals ovulating in evolved populations at 74 and 86 h were consistent with the scenario of significantly accelerated rates of ovulation with evolution (Fig. 3C; 74 h: $t_{24,2} P < 0.01$; 86 h: $t_{21} P < 0.001$). Importantly, at 86 h, evolved hermaphrodites also possessed higher numbers of enlarged oocytes relative to ancestral hermaphrodites (Fig. 3D, $t_{4,8} P < 0.01$), while oocyte size did not evolve (Fig. 3E, $t_{4,9} P = 0.5$). Thus, the concomitant observations of (1) a higher proportion of ovulating individuals and (2) a higher mean number of enlarged oocytes in gonads of evolved hermaphrodites indicate that rates of ovulation, oocyte maturation, and oogenesis became significantly accelerated with evolution. Accelerated oogenesis and ovulation despite a concurrently increased sperm production and developmental delay in the sperm–oocyte switch may therefore have contributed to the evolution of increased self-reproduction at earlier ages.

As for earlier developmental stages, the number of mitotic or meiotic germ cell precursors was unaffected with evolution in chronologically staged hermaphrodites at 68 h (Fig. S2; mitotic: $t_{79,9} P = 0.57$, meiotic: $t_{17,9} P = 0.24$), and at 96 h, the time of reproduction (Fig. S2; mitotic: $t_{57,4} P = 0.55$, meiotic: $t_{15} P = 0.18$). Therefore, although evolved populations displayed faster oogenesis and ovulation, we did not detect a correlated response in oogenic precursor numbers, for example, a reduction of meiotic precursors in evolved populations. This discrepancy may reflect accelerated germ cell proliferation and their meiotic differentiation in evolved populations, which could compensate for the observed increases in oocyte numbers.

EVOLUTION OF DEVELOPMENTAL TIMING IN SOMA AND GERMLINE

To understand how germline proliferation and differentiation integrates with developmental timing across the fixed 96-h life cycle, we quantified somatic progression and reproductive maturation by chronological staging of individuals at 54, 62, and 72 h of the life cycle (Fig. 1B, Table S1). Larval stages at 54 and 62 h were inferred from analysis of vulval cell lineages and morphogenesis (Sulston and Horvitz 1977; Sternberg and Horvitz 1986). Results show that there was no evolutionary response at 54 h but evolved populations were developmentally advanced at the intermediate 62-h time point relative to the ancestral population (Fig. 4A; 54 h: $t_{16,3} P = 0.92$; 62 h: $t_{16,3} P < 0.01$). Specifically, at 54 h, evolved and ancestral hermaphrodites were mostly late L3 larvae, but at 62 h, most hermaphrodites of evolved populations were at the L4-adult transition whereas most hermaphrodites of the ancestral population were still mid-L4 larvae. With experimental evolution, there was thus an acceleration of development from the L3–L4 moult, prior to spermatogenesis,

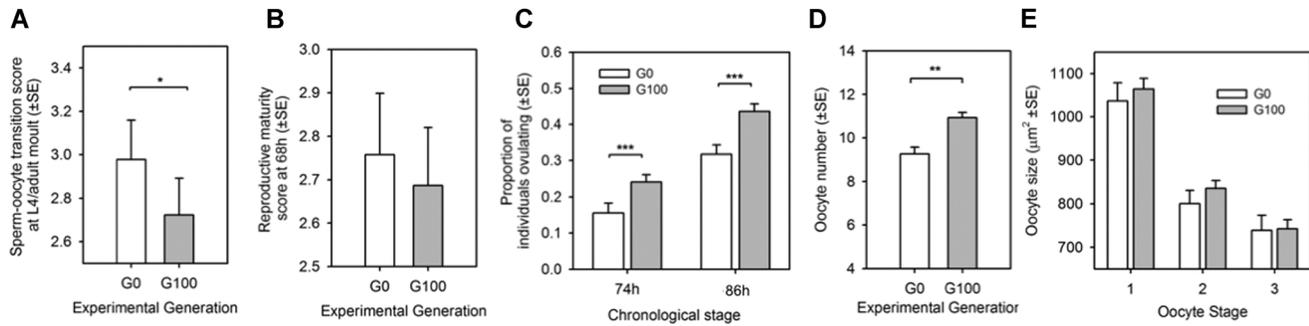


Figure 3. Evolution of the sperm-oocyte switch, oogenesis, and ovulation. (A) Evolved populations showed a significant delay of the sperm-oocyte decision relative to somatic developmental stage (L4/adult moult), based on an index scoring presence of mature spermatids, expression of the early-oogenesis marker RME-2, and presence of oocytes (diplotene-diakinesis stages) in the proximal gonad arm. (B) At 68 h of the life cycle, ancestral and evolved populations did not differ in reproductive maturity (presence of oocytes and/or embryos in uterus). (C) The number of enlarged oocytes was significantly increased at 74 h after experimental evolution. (D) The proportions of individuals ovulating at 74 and 86 h of the life cycle were significantly increased after experimental evolution. (E) Oocyte size (area, $2 \times 10^{-4} \mu\text{m}^2$) did not differ between ancestral and evolved populations. Linear mixed effects and generalized linear mixed effects models used to test for differences among generation 0 (G0) and generation 100 (G100): * P -values < 0.05, ** P -values < 0.01, *** P -values < 0.001. Means and error least-square estimates are shown. See main text for summary statistics.

until the L4-adult moult, when the switch to oogenesis occurs (Fig. 4B).

At 72 h, the great majority of hermaphrodites in both evolved and ancestral populations were young adults that had not yet started laying embryos, and so we distinguished differences in reproductive maturity by scoring the absence/presence of oocytes and counting the number of embryos in utero. At this time point, evolved and ancestral hermaphrodites were on average at the same developmental stage, with most individuals having one to five embryos present in the uterus, which suggests that timing of reproductive maturity, defined as onset of embryo production, did not evolve (Fig. 4A, $t_{16.3} P = 0.25$). This result further confirms the results on an earlier measure of reproductive maturity scored at 68 h (Fig. 3B). Evolution of accelerated developmental time until the switch from spermatogenesis to oogenesis was thus followed by a developmental deceleration until reproductive maturation. Given that the timing of reproductive maturity did not evolve, this suggests that the evolution of increased sperm production—and resulting delay in the onset of oogenesis—was compensated for by the compression of developmental time during the last larval stage.

Discussion

In this study, we have taken advantage of *C. elegans* experimental evolution to characterize the developmental responses that underlie the evolution of increased hermaphrodite early-life self-fertility. In our experiments, heterochrony was defined by differences in relative timing of developmental events in hermaphrodites of the evolved populations when concurrently compared to hermaphrodites of the ancestral population in the context of the imposed 96-h life cycle.

Our analysis uncovered heterochrony not only in gametogenesis but also in the relative progression of germline versus soma (Fig. 4B). We found that ancestral hermaphrodites started spermatogenesis at the mid-L4 stage, switched to oogenesis in the late L4 stage and reached reproductive maturity between 68 and 72 h of the cycle. In contrast, evolved hermaphrodites were at the mid-L4 stage at an earlier time during the life cycle, even though they showed a delayed entry into spermatogenesis. The switch to oogenesis was also delayed in evolved hermaphrodites, likely because of prolonged spermatogenesis; nevertheless, evolved hermaphrodites reached the L4-adult moult at an earlier time point of the life cycle than ancestral hermaphrodites. Eventually, ancestral and evolved hermaphrodites reached reproductive maturity at the same time. Subsequently, observed increases in oogenesis/ovulation (measured at 74 and 86 h) of evolved hermaphrodites can explain their increased early-life fertility relative to ancestral hermaphrodites. Overall, there was little evidence for the evolution of size-related traits (germline cell precursor total numbers, gamete, and embryo size) that could suggest the possible evolution of more efficient resource allocation to both spermatogenesis and oogenesis.

Our results differ from another experimental evolution study using *C. elegans* male-hermaphrodite populations showing that an adaptive shift toward earlier self-reproduction was partly due to accelerated gametogenesis and earlier reproductive maturity (Anderson et al. 2011). It is unclear whether in Anderson et al. (2011) hermaphrodite sex allocation evolved, as evolutionary changes in hermaphrodite cross-fertility or sperm production were not measured. One explanation for the differences in developmental and reproductive timing between studies may be reduced selection

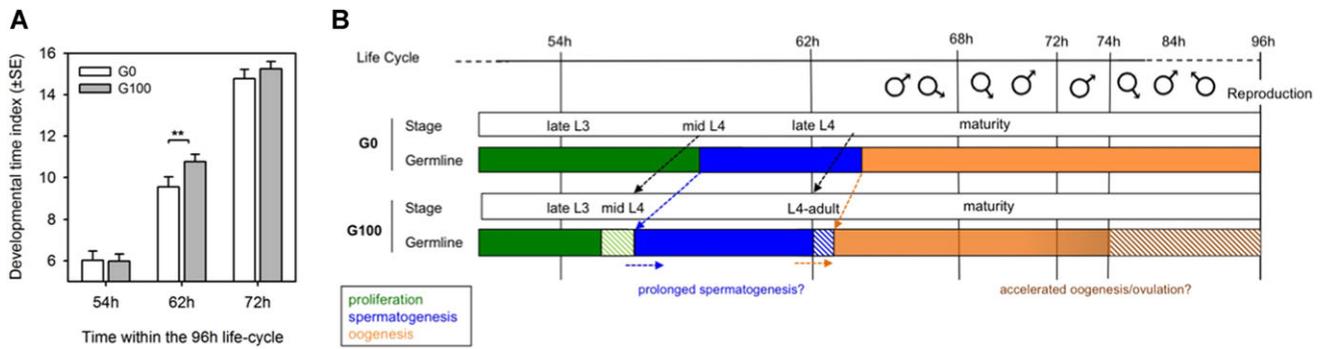


Figure 4. Evolution of developmental timing in soma and germline. (A) Quantification of developmental progression and reproductive maturation by morphological staging of a random set of individuals at 54, 62, and 72 h of the life cycle. In the ancestral population, at 54 h, hermaphrodites were at the developmental stage index 6, corresponding on average to late L3 stage larvae (Table S1), and at 62 h, most hermaphrodites grew to the mid L4 stage and at 72 h they were all early mature adults (indices 14–15). There was no significant evolutionary response in developmental time at 54 or 72 h but evolved populations were developmentally advanced at the intermediate time point of 62 h. At this time point, most individuals of evolved populations had reached the L4/adult moult or early adulthood (index 11), whereas most individuals of the ancestral populations were still in the mid-to-late L4 stages (index 10). Linear mixed effects and generalized linear mixed effects models used to test for differences among generation 0 (G0) and generation 100 (G100): **P*-values < 0.05, ***P*-values < 0.01, ****P*-values < 0.001. Means and error least square estimates are shown. See main text for summary statistics. (B) Schematic outline of evolutionary heterochronic changes in soma versus germline. Ancestral hermaphrodites start spermatogenesis at mid-L4 stage, switch to oogenesis in late L4 stage, reach adulthood shortly after 62 h, and initiate embryogenesis around 68 h of the life cycle. Before reproduction at 96 h, there were therefore about 30 h where males could mate with them (Theologidis et al. 2014). Dashed arrows and boxes indicate the measured developmental changes after 100 generations under the fixed 96-h life cycle. With experimental evolution, hermaphrodites were at the mid-L4 stage at an earlier time, even if they delayed entry into spermatogenesis. The switch to oogenesis was also delayed, possibly because of prolonged spermatogenesis, although they reached the L4 to adult moult earlier than ancestral hermaphrodites and eventually reached maturity (onset of embryogenesis) at the same time (68 to 72 h of the cycle). Oogenesis and ovulation were accelerated until reproduction at 96 h, possibly due to the presence of males, but it is possible that this acceleration started soon after the sperm–oocyte switch.

strength under selfing (Anderson et al. 2011) since male frequencies were maintained at around 40% (Anderson et al. 2010), thus being closer to effective dioecy than in our evolution experiments where males were stably maintained at around 25% (Teotonio et al. 2012). If this was the case, then selection under predominant outcrossing could have resulted in a general acceleration of hermaphrodite gametogenesis and developmental time to maturity, which would have increased hermaphrodite reproductive output under both selfing and outcrossing. Interestingly, however, our results show that soma-germline heterochrony (Fig. 4B) allowed for the evolution of increased sperm production without delaying reproductive maturity—contrary to what is expected from observations in *C. elegans* mutants with increased or decreased self-sperm production (Hodgkin and Barnes 1991; Murray and Cutter 2011). This result shows that an apparent fitness trade-off due to the developmental coupling between spermatogenesis and oogenesis can be minimized through compensatory changes in developmental timing of both germline and soma.

Caenorhabditis elegans show natural genetic variation in diverse hermaphrodite traits associated with sexual functions (Hodgkin and Doniach 1997; Petrella 2014; Pouillet et al. 2015), including genetic variation in temporal expression dynamics of

spermatogenesis- and oogenesis-specific genes (Francesconi and Lehner 2014). However, it remains unknown how such variation at the gene expression level affects particular developmental traits, such as the timing of the sperm–oocyte switch, and which specific alleles may be targeted by selection to generate heterochrony. Transcriptomic analyses of the temporal dynamics of gene expression associated with germline versus somatic development provide many potential candidates (Thoemke et al. 2005; Spencer et al. 2011). While it is possible that such genes directly regulate the central germline sex determination pathway, for example, *fog-2* (Schedl and Kimble 1988), additional genes may also act indirectly on germ cell proliferation and differentiation, through a variety of possible soma-germline interactions, in particular, interactions mediated by the somatic gonad (Ellis 2010; Korta and Hubbard 2010). Whether regulatory changes in genes of the so-called “heterochronic pathway,” whose mutational disruption causes pronounced temporal shifts in the progression of certain somatic *C. elegans* cell lineages (Resnick et al. 2010), could preferentially contribute to the evolution by heterochrony remains questionable, not only with respect to our experimental paradigm but in general. Of note, and as pointed out by Raff (1996), heterochrony need not emerge from changes directly related to

developmental timing itself, such as changes in the expression of “clock” genes. Shifts in the expression thresholds of any gene, for example, may be involved to affect developmental timing. We share this view and consider heterochrony to be best understood as an evolutionary process at the developmental level.

How does heterochrony underlie the adaptive maintenance of partial selfing in our evolution experiments? Teotonio et al. (2012) has shown that evolved populations exhibited improved male competitive performance (mating and cross-fertilization success), possibly due to sexual selection. On the other hand, hermaphrodite early-life fertility was improved specifically under selfing, not under outcrossing with tester males (Carvalho et al. 2014b). This balance between male and hermaphrodite fitness components can therefore explain the maintenance of selfing at intermediate frequencies (partial selfing) from a life-history perspective. It is known that from the time of reaching adulthood until reproduction in our life cycle (from approximately 63–66 h until 96 h), males had ample opportunity to find and mate with hermaphrodites (Fig. 4A, Theologidis et al. 2014). Since after insemination, larger male sperm must crawl through the hermaphrodite uterus to reach the spermatheca, where it actively displaces the smaller self-sperm to ensure cross-fertilization (Ward and Carrel 1979), our results suggest that the increased embryo retention in utero and the increased numbers of self-sperm, without changes in time to reproductive maturity, may have strained cross-fertilization by males. Consistent with this hypothesis, populations with higher self-sperm numbers in the spermatheca and with higher embryo retention were also those where male sperm tended to be larger (Fig. S2). The evolution of developmental timing, without generating differences in the time of reproductive maturity, may have been selected because earlier reproductive maturity would provide more time for males to mate with hermaphrodites. Otherwise, higher outcrossing rates than those observed, perhaps effective dioecy with outcrossing rates of 100%, would have been maintained and that was not the case.

Pollen discounting in angiosperms, an analogous situation to the lack of outcrossing among *C. elegans* hermaphrodites, has been proposed to explain the maintenance of partial selfing (e.g., Holsinger 1991; Porcher and Lande 2005). But there is little evidence that heterochrony can underlie partial selfing through selection for optimal sex allocation although this has been—more or less explicitly—proposed for a long time (Darwin 1877; Mazer et al. 2004; Goodwillie et al. 2005; Weeks et al. 2006; Johnston et al. 2009; Escobar et al. 2011). Besides the studies in *C. elegans* mentioned above (Anderson et al. 2011; Murray and Cutter 2011), a study in the monkeyflower *Mimulus guttatus* has shown that five generations of selection under restricted cross-pollination resulted in the evolution of increased corolla sizes but reduced anther-stigma distances, implying heterochrony in organ

proliferation and differentiation (Roels and Kelly 2011). Similar responses were nevertheless obtained when cross-pollination was not restricted, though not to the same extent, suggesting that heterochrony caused the evolution of more efficient acquisition of resources toward reproduction instead of causing the evolution of sex allocation. In a recent study in the snail *Physa acuta*, 17 generations under restricted outcrossing resulted in reduced developmental time to maturity, although allocation toward male and female functions showed weak responses (Noel et al. 2016). Thus, the few experimental studies suggesting a potential role of heterochrony in the maintenance of partial selfing could not clearly show that heterochrony was due to the evolution of sex allocation.

Overall, our study shows that heterochrony in germline and soma underlies the evolution of higher reproductive performance under selfing to counterbalance the evolution of increased reproductive performance under outcrossing—in our case by the improvement of male fitness components (Teotonio et al. 2012). Heterochrony may therefore be involved in many instances of partial selfing in natural populations with mixed reproduction modes (Goodwillie et al. 2005; Jarne and Auld 2006; Weeks et al. 2006). From a broader perspective, our study shows how subtle, yet complex, heterochrony can underlie life history evolution. Integrating quantitative developmental analysis with experimental evolution can help to elucidate how natural selection operates on the interplay of developmental parameters at the population level, a central goal of evolutionary developmental biology.

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NP, AV, CG, and CB did the assays and preliminary analysis. SC cultured the populations for developmental characterization. HT and CB designed the project, analyzed the data, and wrote the manuscript.

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DATA ARCHIVING

All raw data is provided in Table S2 of the Supporting Information.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Table S1. Criteria used to determine developmental stages.

Table S2. Raw data in Excel format.

Figure S1. Germline progenitor cell counts at 68 and 96 h.

Figure S2. Correlations between male sperm size and hermaphrodite traits.