

1 **Asexual reproduction in male gametophytes of *Laminaria pallida* (Phaeophyceae)**

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1 **Abstract**

2 While female parthenogenesis is common in Laminariales, androgenesis is seldom reported
3 and nearly unstudied. This method of asexual reproduction plays a significant role in the
4 reproduction of many genera. In cultures of *Laminaria pallida*, isolated male gametophytes
5 were observed to develop egg-like structures that further developed into juvenile sporophytes.
6 This mechanism of asexual reproduction and the dysmorphia of produced sporophytes suggest
7 an incomplete suppression of femaleness during sexual determination of gametophytes, leading
8 to a partial hermaphroditism. The trait is clearly inherited, as all studied unialgal strains
9 descended from a unique sporophyte but showed significant variability between individual
10 strains. Successful gametogenesis and sexual reproduction were not prevented but may be
11 impeded by androgenetic ability. Further investigations into the genetics and maturation of
12 androsporophytes will help to estimate the potential effects of this mechanism on natural
13 populations.

14

1 **1. INTRODUCTION**

2 True kelps of the order Laminariales are key habitat forming species in cold to temperature
3 rocky coastal ecosystems, providing important ecological and economic services (Hop et
4 al., 2012; Oliver et al., 2018; Smale, 2020). *Laminaria pallida* (Greville), distributed
5 throughout the southern West coast of Africa, is the sole habitat-forming species in
6 Namibia and one of two dominant kelps in western South Africa (Molloy, 1990; Rothman
7 et al., 2017). Populations located at the northern distribution boundary are generally
8 scattered (Rothman, 2015) and are characterised by high inbreeding rates and low genetic
9 diversity (Assis et al., in prep).

10 Kelps are characterised by a heteromorphic life cycle alternating between diploid
11 (sporophytes) and haploid (meiospores, gametophytes) stages (Boney & Luning, 1991; Liu
12 et al., 2017). Macroscopic sporophytes undergo meiosis to release meiospores, which
13 differentiate into either male or female gametophytes. Female gametophytes form oogonia
14 and release eggs and the pheromone lamoxirene, which triggers sperm release from males
15 and attracts it chemotactically (Maier et al., 2001). Fecundation leads to the formation of a
16 microscopic sporophyte, closing the haplodiplontic life cycle.

17 External fertilisation strongly decreases chances of reproductive success in gametophytes
18 (Serrao et al., 1996). Female parthenogenesis is a common mode of asexual reproduction
19 in kelps and is well understood (Lewis et al., 1993; Motomura, 1991; Oppliger et al., 2007).
20 Unfertilized eggs develop into sporophytes of various ploidy, which tend to exhibit
21 morphological anomalies and high juvenile mortality (Pang & Tai, 1976). Mature
22 parthenosporophytes release meiospores which differentiate exclusively into female
23 gametophytes, which are generally fertile for sexual reproduction (Lewis et al., 1993).
24 Parthenogenetic ability is linked to genetic material, varies significantly between female
25 gametophytes and is transmitted to further generations (Jixun et al., 1993; Shan et al.,

1 2013). In brown algae of the genus *Ectocarpus*, parthenogenesis was linked to the sex locus,
2 where genetic recombination is extremely rare in algae (Coelho et al., 2019; Mignerot et
3 al., 2019). This ability is therefore unlikely to be passed onto male gametophytes.

4 Androgenesis, the asexual reproduction of males, is common in plants where it leads to
5 healthy, haploid individuals (Chupeau et al., 1998; Seguí-Simarro, 2010). It is also
6 described in several genera of the class Phaeophyceae (*Colpomenia*: Clayton, 1979;
7 *Scytoniphon*: Clayton, 1980; Desmarestia: Ramirez et al., 1986). In most cases, juvenile
8 sporophytes emerged from unfused spermatozoids, similarly to female
9 parthenosporophytes (Lewis, 1996; Yamagishi & Kogame, 1998). In *Perithalia caudata*,
10 large structures evolved directly from somatic cells of male gametophytes before becoming
11 sporophytes (Müller et al., 1985). In Laminariales, androgenesis was only reported in
12 *Laminaria japonica* (Fang, 1983) and a South African population of *L. pallida* (Coelho et
13 al., 2019), despite attempts to record it in other species (Oppliger et al., 2007). The genetic
14 and physiological mechanisms underlying androgenesis in algae are greatly understudied,
15 and opportunities to experiment on asexually reproductive males are scarce. However, it is
16 important to evaluate the effects of androgenesis on male gametophytes and the
17 sporophytes they yield, both sexually and asexually. In plants where androgenesis yields
18 fit individuals, the associated alleles often spread to fixation, which could have dramatic
19 effects on wild populations (McKone & Halpern, 2003). Additionally, successful
20 androgenesis can be used to significantly improve methods of genetic engineering for
21 aquaculture purposes (Islam & Tuteja, 2012).

22 We observed some male gametophytes of *Laminaria pallida* undergoing asexual
23 reproduction in isolated cultures. Gametophytes were placed in optimal reproductive
24 conditions, both isolated and in crosses with female gametophytes. Gametogenesis and
25 androgenesis were measured in isolated and crossed gametophytes to compare the ability

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1 in different unialgal strains. Related female unialgal strains were also observed to test
2 potential effects on females. In crosses, reproductive success was measured to estimate
3 potential impacts of the androgenetic ability on the sexual reproduction of males. This study
4 aims to investigate androgenesis to gain a foundation understanding of its effects and pave
5 the way for further genetic and ecophysiological studies.

6

7 **2. MATERIALS AND METHODS**

8 **2.1 Experimental design**

9 Pre-experimental observations showed several gametophyte male strains obtained from a
10 single sporophyte (Spo. A) undergoing androgenesis. To assess the genetic variation in
11 androgenetic ability, gametogenesis was induced in single-strain cultures of male
12 gametophytes obtained from Spo. A and the proportion of asexually reproductive males
13 recorded after 20 days. Female gametophytes from Spo. A were also investigated to
14 determine whether their parthenogenetic ability differed from previously published data.
15 The reproductive ability of androgenetic male gametophytes was evaluated by measuring
16 their reproductive success when crossed with females obtained from other sporophytes for
17 26 days.

18

19 **2.2 Algal material**

20 Mature sporophytes of *Laminaria pallida* were sampled from Swakopmund,
21 Namibia (coordinates: 22.672 S, 14.522 E) in July 2019. Sori were cleaned and meiospores
22 from each sporophyte were released separately in sterile seawater. After spore germination,
23 several unique male and female gametophytes were isolated to establish single-sex unialgal
24 cultures for each sporophyte individual. Cultures were maintained in a vegetative state in
25 sterile half-strength Provasoli enriched seawater (PES, Provasoli, 1968) at 12°C under 3

1 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of red light and 16h:8h light:dark (L:D) cycle in a climate-controlled
2 chamber (Fitoclima S600, Aralab, Lisbon, Portugal). Sterile artificial seawater (Tropic
3 Marin Sea Salt, Wartenberg, Germany) with a salinity of 34 ± 1 ppm was used for
4 maintenance and all experiments. The culture medium was changed monthly until the
5 beginning of the experiment (ca. 20 months).

6

7 **2.3 Gametogenesis and apomixis in isolated strains**

8 Eight unialgal male cultures (σ A.n) and three unialgal female cultures (φ A.n) were used
9 for this experiment. Each culture of vegetative gametophytes was gently ground using a
10 pestle and mortar, sieved and diluted to produce a stock solution of gametophyte filaments
11 with lengths $\leq 100 \mu\text{m}$. From each stock solution, the volume needed to achieve densities
12 of ~ 400 gametophytes cm^{-2} was added to Petri dishes (5.3cm in diameter, height 1.5cm)
13 containing 10ml of half-strength PES. Four replicate Petri dishes were used for each strain.
14 (11 strains \times 4 replicates = 44 Petri dishes in total). The gametophytes were allowed to
15 recover from the mechanical stress induced by fragmentation for 2 days at 14°C under 3
16 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of red light in a 16h:8h L:D cycle. After this period, the gametophytes
17 were transferred to 18 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of white light to induce gametogenesis. The
18 temperature of 14°C was chosen as it provides good gametogenetic conditions (Martins et
19 al., 2019; tom Dieck & de Oliveira, 1993). The culture medium was changed every 10 days
20 by replacement of 5ml of half-strength PES.

21

22 **2.3.a. Gametogenesis and asexual reproduction ability**

23 The relative occurrence of three ontogenetic stages (vegetative, antheridia, sporophytes
24 attached for males and vegetative/oogonia, eggs released and sporophytes attached for
25 females) was measured after 20 days of gametogenic conditions in a minimum of 200

1 gametophytes per replicate using a Zeiss Observer D1 inverted microscope (Carl Zeiss
2 MicroImaging GmbH, Göttingen, Germany). The most advanced developmental stage was
3 assigned for each gametophyte. Sporophytes were considered as soon as the first cell
4 division was visible in the zygote.

5

6 **2.4 Reproductive success in crosses**

7 The same eight unicellular cultures of angrogenetic male gametophytes (σ A.n) and three
8 control single-strain cultures of male gametophytes obtained from another sporophyte
9 (σ C.n) were crossed with a pool of six female gametophyte strains obtained from three
10 separate sporophyte (2 females strains per sporophyte) to avoid possible selfing effects. All
11 male strains and the female pools were ground as described in part 2.3. Crosses were
12 obtained by combining one single-strain male solution with female pool stock solution into
13 Petri dishes (5.3cm in diameter, height 1.5cm) containing 10ml of half-strength PES to
14 achieve total densities of ~400 gametophytes cm^{-2} . Four replicate Petri dishes were used
15 per cross (11 crosses \times 4 replicates = 44 Petri dishes in total). The gametophytes were
16 allowed to recover from the mechanical stress induced by fragmentation for 2 days at 14°C
17 under 3 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of red light in a 16h:8h L:D cycle. After this period the
18 gametophytes were transferred to 18 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white light to induce
19 gametogenesis, and the culture medium was changed every 10 days by replacement of 5ml
20 of half-strength PES.

21

22 **2.4.a. Male gametogenesis and asexual reproduction ability**

23 The relative occurrence of three ontogenetic stages (vegetative, antheridia, sporophyte
24 attached) was measured in σ A crosses as described in part 2.3.a. after 20 days in
25 gametogenetic conditions.

1

2 **2.4.b. Reproductive success**

3 Reproductive success was evaluated through the density of sporophytes attached to female
4 gametophytes after 26 days, evaluated by counting sporophytes in ≥50 fields of view (Zeiss
5 Observed D1 inverted microscope, 100× magnification) per replicate. Sporophyte densities
6 were statistically compared between crosses using a one-way ANOVA test in the R
7 Statistical Environment ((R Core Team, 2021). Data was tested for normality within groups
8 using the Shapiro-Wilk test and homoscedasticity using Levene's test, and a post-hoc
9 Tukey test was conducted to determine differences between crosses ($p < 0.05$).

10

11 **2.5 Pictures**

12 At all stages of development, pictures were taken using a Nikon D90 camera (Nikon,
13 Tokyo, Japan) mounted on a Zeiss Observer D1 inverted microscope.

14

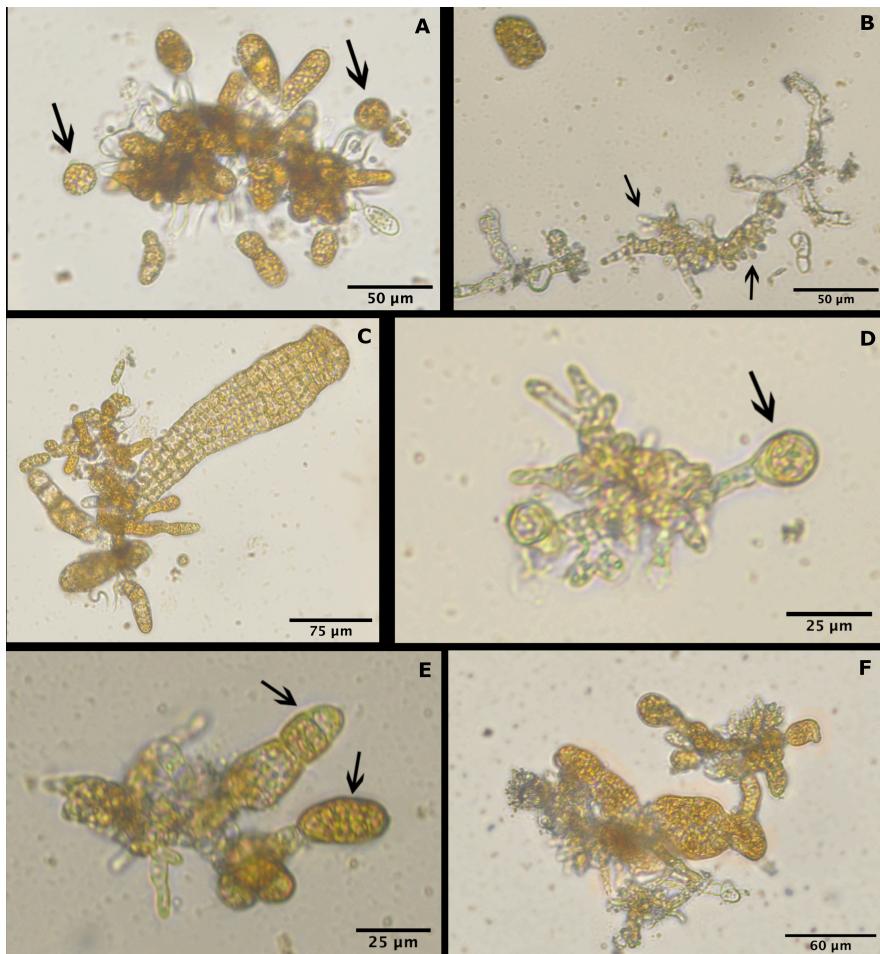
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16 **3. RESULTS**

17 **3.1 Observations/pictures of androgenesis**

18 Gametophytes obtained from Sporophyte A displayed normal morphology, both in males and
19 females. Isolated females developed oogonia and released eggs when placed in gametogenetic
20 conditions (Fig. 1A). Similarly, males developed antheridia in gametogenetic conditions, both
21 when isolated and in crosses (Fig. 1B). Females crossed with ♂A developed healthy
22 sporophytes with normal morphologies (Fig. 1C). Both when isolated and in crosses, ♂A
23 gametophytes developed growths visually similar to eggs observed in female gametophytes
24 (Fig. 1D), which further developed into multicellular sporophytes (Fig. 1E). Upon developing

1 further, these sporophytes assumed abnormal, bulky morphologies (Fig. 1F) akin to
2 parthenosporophytes observed in isolated females.



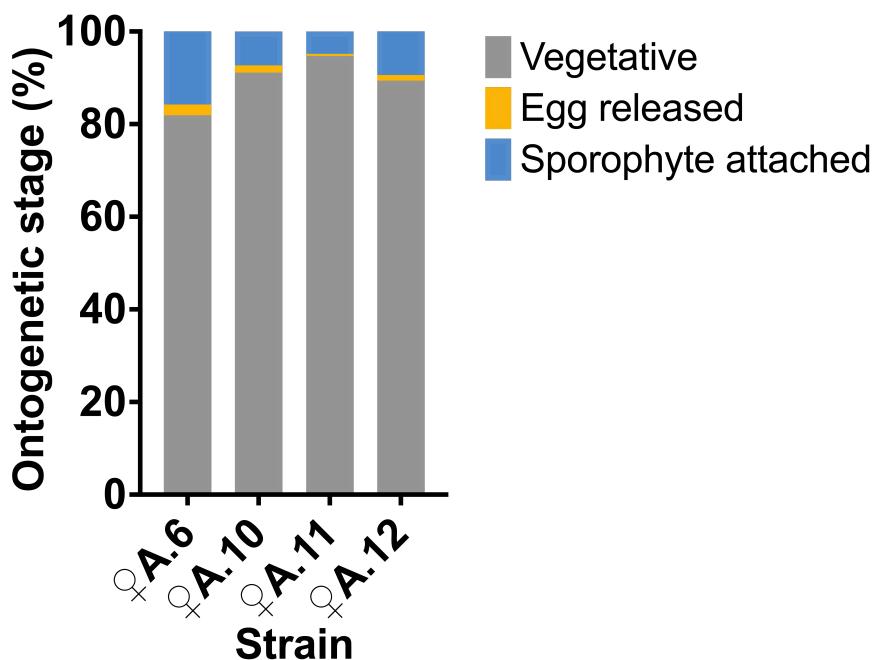
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4 **Figure 1: Gametophytes of *Laminaria pallida*.** A. Isolated ♀ A gametophyte. Arrows point
5 to released eggs. B. Isolated ♂ A gametophyte. Arrows point to antheridia; a detached
6 dysmorphicous sporophyte is visible in the upper left corner. C. Healthy sporophyte in cross
7 between ♂ A and female pool. D. Isolated androgenetic male with visible antheridium. Arrow
8 points to egg-like structure. E. Isolated androgenetic male. Arrows point to cell division in

1 juvenile sporophyte. F. Isolated androgenetic male with multiple dysmorphous sporophytes
2 attached and visible antheridia.

3

4 **3.2 Isolated female gametogenesis**

5 Isolated ♀ A gametophytes underwent limited gametogenesis, with the percentage of
6 reproductive females after 20 days ranging between 5-18% depending on crosses.
7 Parthenogenesis was extensive in all crosses, with a minimum of 80% of reproductive females
8 having a sporophyte attached.

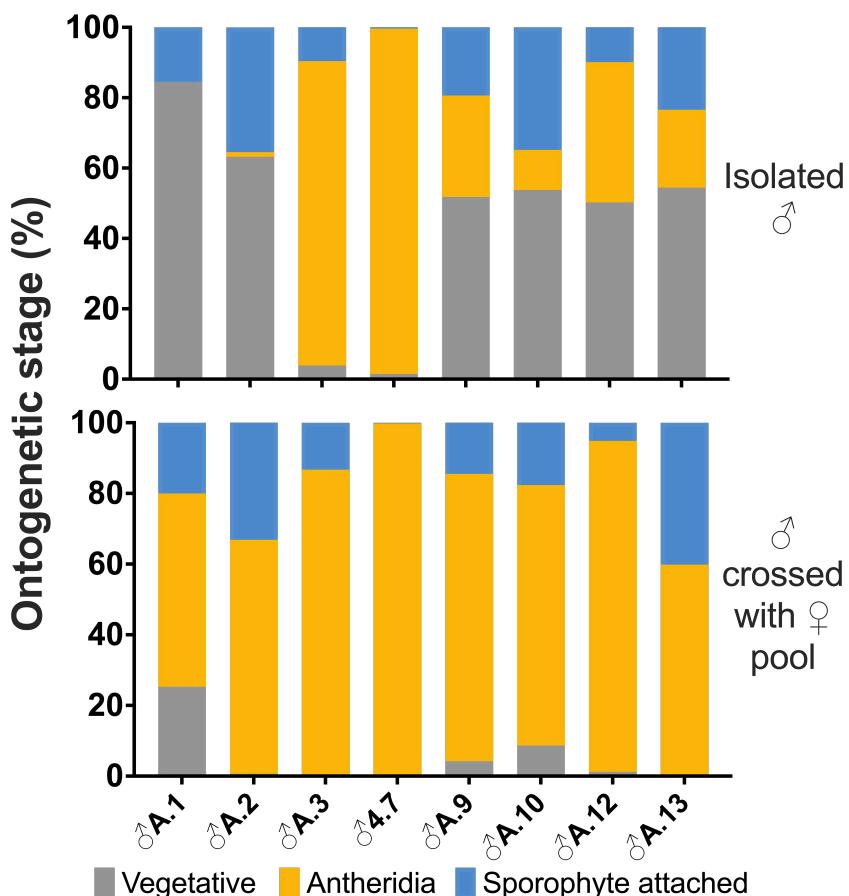


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10 **Figure 2: Gametogenesis development of different ♀ A unialgal strains of *L. pallida* after
11 20 days in gametogenic conditions.** 100% stacked column chart with means of each
12 ontogenetic stage (n=4). SE-values are omitted for clarity.

13

1 **3.3 Male gametogenesis**

2 Sporophytes were attached to male gametophytes in all σ A strains except σ A.7, which
3 showed extremely limited to no androgenesis (Fig. 3). Proportions of male gametophytes with
4 at least one sporophyte differed between strains but were generally similar for each strain
5 whether isolated or crossed with females. Males in crosses developed more antheridia than
6 isolated males after 20 days, except for strains σ A.3 and σ A.7, which showed the lowest
7 androgenetic abilities.

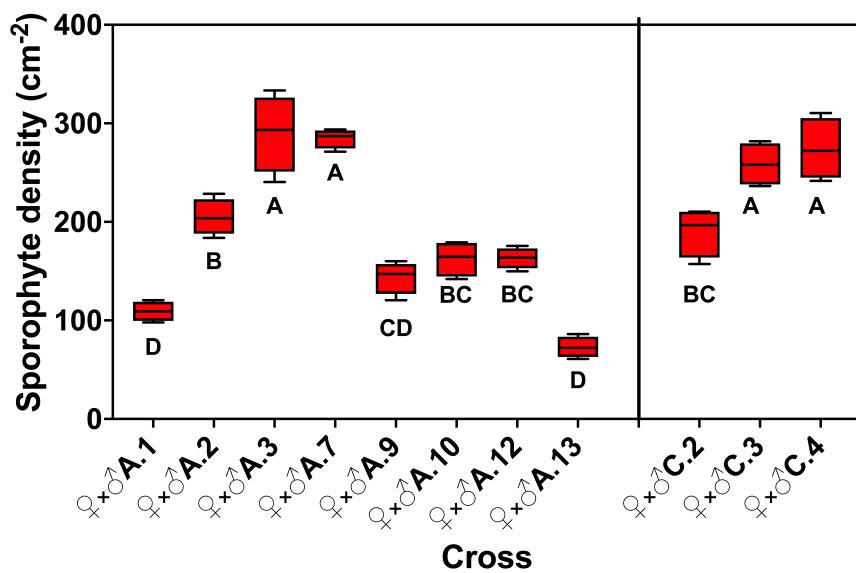


1 **Figure 3: Effect of isolation/crossing on the male gametogenesis development of different**
2 **androgenetic unialgal strains of *L. pallida* after 20 days in gametogenic conditions.** 100%
3 stacked column charts with means of each ontogenetic stage (n=4). SE-values are omitted for
4 clarity.

5

6 **3.4 Reproductive success**

7 All ♂A gametophyte strains successfully reproduced in crosses (Fig. 4). Crosses with all
8 strains except ♂A.1 and ♂A.13 had sporophyte densities statistically equivalent to at least
9 one control cross with non-androgenetic males. Crosses with strains ♂A.3 and ♂A.7 had
10 significantly higher sporophyte densities after 26 days than crosses with all other ♂A.



11

12 **Figure 4: Reproductive success of different unialgal male strains of *L. pallida* with a**
13 **female pool after 26 days in gametogenic conditions.** Sporophyte density, box plots with
14 medians, boxes for 25th and 75th percentiles and whiskers indicating min and max values (n=4).

1 Panels separate test males from androgenetic lines (σ A) from control males (σ C). Different
2 letters indicate significant differences between crosses ($p < 0.05$). See Table 1 for statistics.

3

4 **Table 1:** Anova for the effects of male strain on the density of sporophytes attached to females
5 after 26 days in gametogenic conditions. The post-hoc results are presented in Fig. 4.

Factor	df	SS	MS	F	P
Male strain	10	217963	21796	47.52	<0.001
Residual	33	15137	459		

6 Significant main effects are highlighted in bold. df: degrees of freedom; SS: sum of squares;
7 MS: mean sum of squares.

8

9 DISCUSSION

10 Androgenesis was observed for the first time in Laminariales other than *L. japonica* (Laviolette
11 et al., 1967; Fang, 1983) and another population of *L. pallida* (Coelho et al., 2019). As an
12 ability ranging from anecdotal in many species to the main mode of reproduction for some
13 genera (e.g. *Corbicula*, Pigneur et al., 2012), it is key to understand its effects and potential
14 implications for *L. pallida*. In most species, androgenesis proceeds by elimination of female
15 genetic material after fecundation, and while offspring are nuclear paternal clones, a female is
16 required for the process (Normark, 2009). However, we recorded androgenesis both when
17 males were isolated and in cultures with females. Androgenesis in *L. pallida* has been referred
18 to as male parthenogenesis (Coelho et al., 2019). Our observations strongly corroborate such a
19 description. Males seemed to produce their own egg-like structures which further developed
20 into sporophytes, a mechanism reminiscent of parthenogenesis commonly observed in
21 Laminariales female gametophytes (e.g., Oppliger et al., 2007). The dysmorphia observed in
22 androsporophytes is also similar to the bulky shapes of anomalous parthenosporophytes, which

1 are characterised by high juvenile mortality (Pang & Tai, 1976). Survival of
2 parthenosporophytes of *L. japonica* is linked to their ploidy, with haploid specimens commonly
3 able to grow and produce female gametophytes. (Lewis et al., 1993). Androsporophytes
4 recorded in *L. japonica* showed similar dysmorphia, inhibited growth and never became
5 reproductive (Laviolette et al., 1967).

6

7 **4.1 Androgenesis is genetically inherited and variable**

8 All gametophytes exhibiting androgenesis were obtained from a single sporophyte, pointing to
9 an inherited ability. The sporophytes sampled come from a highly inbred population in
10 Northern Namibia (Assis et al., in prep.), while similar observations were made in
11 gametophytes obtained from a South African population (Coelho et al., 2019). Additionally,
12 all unicellular male strains used in our experiment showed some degree of androgenetic ability.
13 While androgenesis is clearly inherited, its presence in two widely distant populations suggests
14 the activation of a ubiquitous gene rather than a new mutation. U/V haploid systems are
15 characterised by non-recombining sex determining regions, with symmetrical evolution and
16 low degeneration of the U and V chromosomes (Immler & Otto, 2015). In the Phaeophyceae
17 model genus *Ectocarpus*, dominance of the V (male) chromosome was observed, suggesting
18 that the default gender of gametophytes is female, which is repressed by male expression
19 (Ahmed et al., 2014). Defective suppression of femaleness may lead to expression of both male
20 and female characteristics in the gametophyte, as has been recorded in *Undaria pinnatifida* (Li
21 et al., 2014). The monoecious gametophytes developed both oogonia and antheridia and were
22 able to undergo reproduction through selfing, and fecundation, and produced healthy, fertile
23 sporophytes. In our case, observations point to a reproduction system closer to female
24 parthenogenesis, suggesting partially hermaphroditic gametophytes, where female eggs
25 develop but may not be fertilisable by spermatozoids. The significant variability in

1 androgenesis between unialgal strains is likely to be linked to differences in suppression of
2 femaleness, leading to varied levels of expression of the female parthenogenic ability.

3

4 **4.2 Androgenetic males undergo successful sexual reproduction**

5 All androgenic strains were fertile in crosses with females. The two strains with lowest
6 androgenetic ability yielded significantly more sporophytes than other males, suggesting that
7 female expression may decrease the male gamete production in gametophytes. However, in
8 other strains there was no negative correlation between the percentage of androgenetic
9 gametophytes and reproductive success. The two processes may be mostly independent, as
10 there was little to no difference in androgenetic activity between isolated and crossed cultures,
11 despite a significant increase in antheridia formation. Similarly, monoecious gametophytes of
12 *U. pinnatifida* had remarkably high fertility both in males and females (Li et al., 2014). It
13 appears that potential deactivation of female suppression comes at a limited cost to male
14 gametogenesis. In non-hermaphrodites, dominant androgenesis can lead to heavily male-biased
15 sex ratios and eventual extinction, and the conservation of strong ability for sexual reproduction
16 is positive (Schwander & Oldroyd, 2016).

17

18 **4.3 Androgenetic ability may promote parthenogenesis in related females**

19 Female gametophytes obtained from sporophyte A displayed much higher parthenogenic
20 ability than previously reported. In similar conditions (15°C, 15 µmol photons m⁻² s⁻¹), isolated
21 *L. pallida* gametophytes had almost no attached sporophytes after 33 days in gametogenic
22 conditions (Martins et al., 2019). Parthenogenesis in *Ectocarpus* is controlled by a locus in the
23 sex-determining region of the U chromosome (Mignerot et al., 2019). Due to non-
24 recombination, a decrease in regulation of parthenogenesis may be masked by successful
25 sexual reproduction and be transmitted to offspring (Immler & Otto, 2015).

1 Further studies examining the growth and reproduction of androsporophytes, as well as the
2 genetic material of both gametophytes and sporophytes would help understand the origins and
3 potential implications of male asexual reproduction in *L. pallida*.

4

5

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