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Evolutionary biology

A personal cost of cheating can stabilize reproductive altruism during the early evolution of clonal multicellularity

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Understanding how cooperation evolved and is maintained remains an important and often controversial topic because cheaters that reap the benefits of cooperation without paying the costs can threaten the evolutionary stability of cooperative traits. Cooperation—and especially reproductive altruism—is particularly relevant to the evolution of multicellularity, as somatic cells give up their reproductive potential in order to contribute to the fitness of the newly emerged multicellular individual. Here, we investigated cheating in a simple multicellular species—the green alga *Volvox carteri*, in the context of the mechanisms that can stabilize reproductive altruism during the early evolution of clonal multicellularity. We found that the benefits cheater mutants can gain in terms of their own reproduction are preempted by a cost in survival due to increased sensitivity to stress. This personal cost of cheating reflects the antagonistic pleiotropic effects that the gene coding for reproductive altruism—*regA*—has at the cell level. Specifically, the expression of *regA* in somatic cells results in the suppression of their reproduction potential but also confers them with increased resistance to stress. Since *regA* evolved from a life-history trade-off gene, we suggest that co-opting trade-off genes into cooperative traits can provide a built-in safety system against cheaters in other clonal multicellular lineages.

1. Introduction

The contribution of cooperation to life on Earth—from its inception to the emergence of complex societies—is indisputable [1–3]. However, understanding how cooperation evolved and is maintained remains a challenge for both evolutionary and behavioural sciences, as cheaters [4] that reap the benefits of cooperation without paying the costs can increase their individual fitness and thus threaten the evolutionary stability of cooperative traits [1,5–7]. Cooperation is particularly relevant to evolutionary transitions in individuality (e.g. the evolution of multicellularity and eusociality), since a subset of individuals give up their reproductive potential in order to contribute to the fitness of the newly emerged higher level individual [1,6,8]. This form of reproductive altruism is best typified by somatic cells in multicellular organisms and sterile workers in eusocial insects.

Most obligate multicellular lineages—such as animals, plants, fungi and brown/red/green algae—are clonal; that is, all cells in the body are ultimately derived from a single cell (spore or zygote) or a small group of cells. This high level of relatedness is thought to promote and maintain cooperation between reproductive and somatic cells, and among somatic cells themselves [6,9–11]. However, mutations that increase the fitness of somatic cells can still occur [7]. In animals, these mutations can result in cancer, and multiple mechanisms

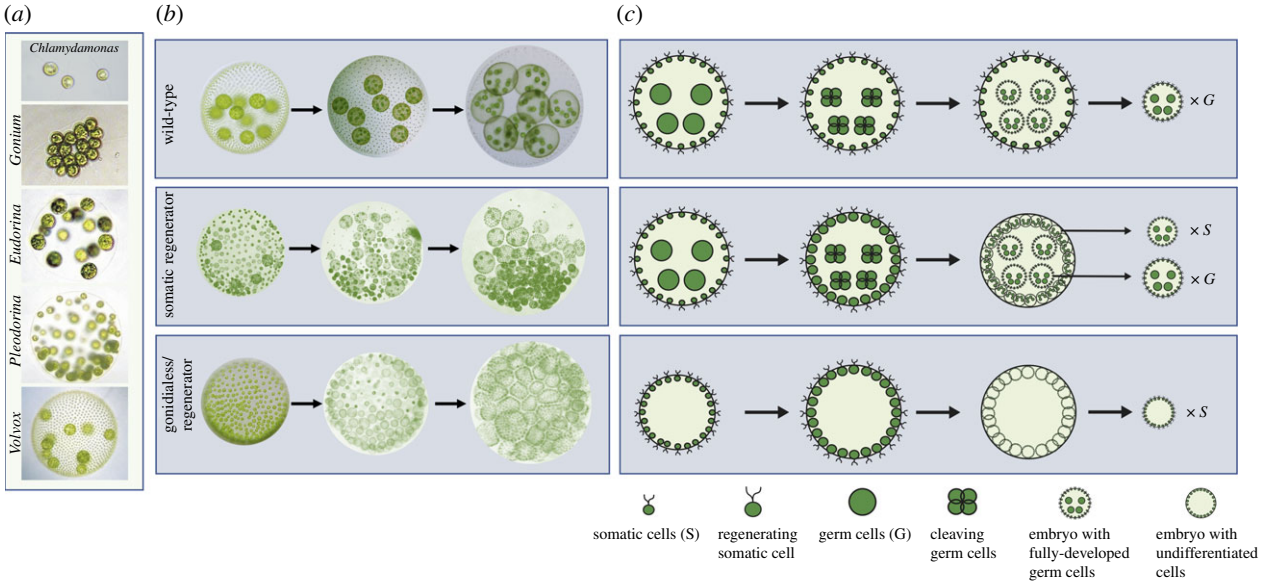

Figure 1. (*a*) Representative volvocine green algae that exhibit different degrees of cooperation (see electronic supplementary material, figure S1 for additional information). (*b*) Micrographs of a *Volvox carteri* wild-type (Eve)—showing the development of gonidia into embryos; a somatic regenerator mutant (regAMN)—in which, in addition to germ cells, somatic cells grow and develop into embryos; and a gonidialess/regenerator mutant (dmSM625-31) – with all its cells developing into embryos. (*c*) Schematic representations of the life cycles of a wild-type (top), somatic regenerator (middle) and gonidialess/regenerator (bottom) strain showing differences in development and number of offspring. Specifically, while in the wild-type, all offspring is derived from germ cells, in regenerator mutants there are two types of offspring—small individuals developed from regenerating somatic cells and larger offspring from germ cells; on the other hand, in gonidialess/regenerator mutants, all cells produce offspring. '*x* G' and '*x* S' indicate that the offspring is a multiple of the number of germ and somatic cells, respectively.

have evolved to suppress/police/punish such cheaters or limit their success [12,13]. However, the impact of cheaters that regain full reproductive potential (both immortality and totipotency) on the stability of reproductive altruism during the early evolution of clonal multicellularity has not been addressed.

The volvocine green algae are an ideal model system to investigate the general principles and genetic basis underlying the early evolution of multicellularity and somatic cell differentiation [14,15]. They are a group of freshwater flagellated algae comprising closely related taxa that exhibit different levels and degrees of cellular cooperation, including single-celled organisms (e.g. *Chlamydomonas*), simple multicellular individuals in which all cells cooperate and benefit (synergistic cooperation; e.g. *Gonium* and *Eudorina*), and multicellular organisms with specialized germ and somatic cells (reproductive altruism)—such as *Volvox carteri* (figure 1*a*; electronic supplementary material, figure S1).

In *V. carteri* (figure 1*a*), the somatic cells are terminally differentiated and express the most extreme form of altruism—i.e. reproductive sterility. By not dividing, the 2000–4000 somatic cells maintain the motility of the individual (essential for survival) while the 10–16 non-flagellated reproductive cells (gonidia) grow extensively and then divide 11–12 times to produce fully formed juveniles that are released and start a new generation (figure 1*b*,*c*). The expression of the altruistic behaviour is determined by a single gene, *regA*, which is a *bona fide* altruism gene [16]. *regA* encodes a transcription factor (RegA) expressed only in cells that fall under an 8 µm threshold size at the end of embryogenesis and then develop into somatic cells [17,18]. A series of embryonic asymmetric cell divisions ensure that some cells remain large, do not express *regA* and differentiate into gonidia [19]. RegA is thought to act by suppressing the expression of nuclear-encoded chloroplast proteins [20], which in turn will prevent the growth (dependent on photosynthesis) and division (dependent on growth) of somatic cells and, thus, their direct representation into the offspring.

As expected for an altruism gene, the loss-of-function mutations in *regA* completely abolish the altruistic behaviour and restore the reproductive potential of somatic cells in *V. carteri* [21]. In these *regA* mutants, known as somatic regenerators, the small cells initially act like somatic cells, but then de-differentiate, lose flagella and re-differentiate as gonidia that fully develop into free-living offspring (figure 1*b*,*c*; electronic supplementary material, figure S2). Somatic regenerator mutants can incur additional mutations in a set of genes known as *gls* (*g*onidia*l*e*s*s) involved in the embryonic asymmetric divisions [22]. In such mutants, all cells start as small somatic-like cells but then all grow and produce offspring (figure 1*b*,*c*). These gonidialess/somatic regenerator mutants lack differentiated cells and are reminiscent of *Eudorina* species (figure 1*a*) that diverged before the evolution of soma in the lineage leading to *V. carteri*.

By reneging their altruistic/somatic fate and reproducing themselves, *regA* mutant cells are *de facto* cheaters, analogous to cheater workers laying eggs in some eusocial insect colonies [23]. Specifically, individual *regA* mutant cells within the multicellular individual increase their direct fitness by producing their own offspring. Once they hatch, these free-living multicellular offspring carry the cheater mutation in all their cells. Since both the somatic and germ cells can reproduce, such multicellular cheaters can produce much higher numbers of offspring relative to the wild-type—i.e. a *group-level reproductive benefit* (figure 1*c*). Furthermore, since their cells still express some ancestral synergistic cooperation traits such as adhesion and production of the extracellular matrix, these cheaters can also maintain some of the *group-level survival benefits* of multicellularity (i.e. increased size and motility). Consequently, in nature, *regA* mutants should

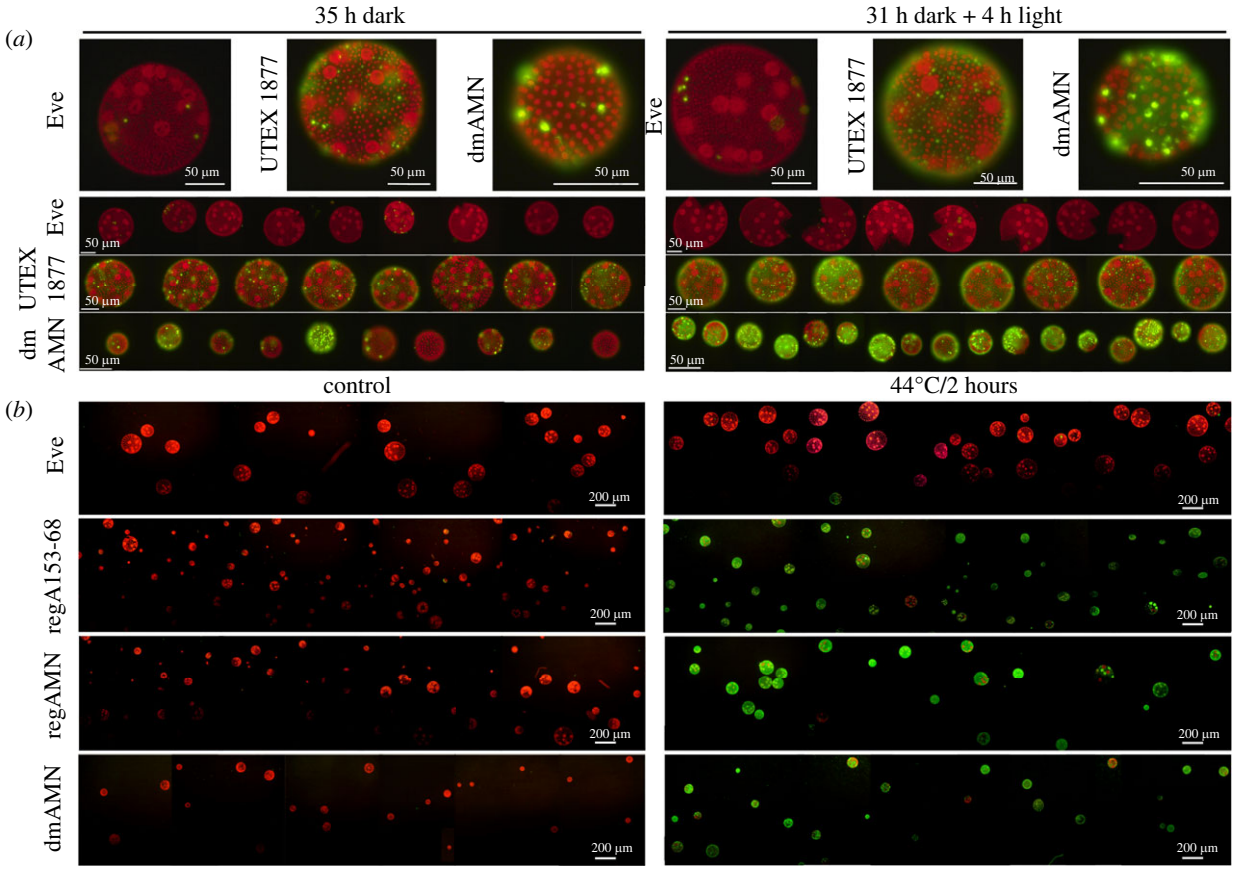

Figure 2. Differences in sensitivity to (*a*) extended dark, and dark and light stress and (*b*) heat stress, between a wild-type *Volvox carteri* strain (Eve) and several somatic regenerator (UTEX 1877, regA 153-68 and regAMN) and gonidialess/regenerator (dmAMN) mutants. Red (chlorophyll autofluorescence) and green (Sytox Green) denote live and dead cells, respectively. See electronic supplementary material, figures S4–S7 for additional mutants, stress conditions and images at higher magnification.

outcompete the wild-type. Yet, that does not seem to be the case, although regenerator mutants can occur spontaneously with high frequency in the laboratory [24,25].

One possible explanation for this observation is that the motility of the multicellular cheaters is negatively affected while cells regenerate because, as in most flagellated cells (including in animals), cell division and flagellar motility cannot take place simultaneously (the 'flagellation constraint'; [26]). Therefore, the loss of flagella during reproduction coupled with the increase in drag due to the mass of the additional somatic embryos could impose a strong *group-level survival cost* [27]. However, these motility costs are likely to have been less significant during the early evolution of somatic cells, which is thought to have occurred in species with as few as 32–64 cells, similar to the extant *Eudorina*/*Pleodorina* species (figure 1*a*). In these species, very few cells differentiate into somatic cells, and thus the motility cost associated with loss of flagella in cheaters would have been much smaller. In such circumstances, additional mechanisms might have been required to stabilize the early evolution of somatic cells.

Recently, using a *gls*/*regA* mutant that expresses a non-functional RegA protein, we showed that in addition to being expressed during development, *regA* could also be induced environmentally, in response to a combination of extended dark and light [28]. Unexpectedly, the same conditions that induced *regA* ultimately resulted in the programmed cell death of many of the small somatic-like cells of the mutant but not of the wild-type somatic cells. Based on this observation we suggested that, in addition to establishing the somatic cell fate, RegA can confer resistance to stress [28,29]. If that were the case, somatic-like cells in *regA* mutants should generally be more sensitive to stress.

This hypothesized increased sensitivity to stress could impose a *cell-level survival cost* on cheaters that can pre-empt their benefit of regaining reproductive potential, irrespective of other potential group-level survival costs. Furthermore, cheaters could be purged out by cell-level selection within the parental wild-type individual before they can reproduce. As volvocine algae live in fluctuating environments, this *personal cost of cheating* might have played an important role in stabilizing reproductive altruism during the early evolution of soma. To address this hypothesis, in this study, we compared the sensitivity of a wild-type *V. carteri* strain and several somatic regenerator and gonidialess/regenerator mutants on different genetic backgrounds, to various types of stress (e.g. extended dark, dark and light, and heat).

2. Material and methods

The specific characteristics of the *V. carteri* strains, including the mutants and mutations described in this study, are summarized in electronic supplementary material, table S1 and figure S3. Experimental cultures (100 individuals per sample; several independent experiments) were exposed to various combinations and types of stress, including 35 h of dark or 31 h of dark followed by 4 h of light (as in [28]), and 42.5–44°C for 1–2 h (figure 2; electronic supplementary material, figures S4–S7). Cell death was assessed with the Sytox Green viability dye and fluorescence

microscopy (using a FITC filter) and collages of random images at low magnification were generated (figure 2); higher magnification images are included in the electronic supplementary material. Based on estimated percentages of dead cells, individuals were grouped in several categories, and chi-square tests of independence [30] were performed to examine the relation between strains and sensitivity to stress (see electronic supplementary material for additional detail and data).

3. Results

(a) Somatic regenerator mutants are sensitive to extended dark and light stress

We have previously found that a combination of 31 h of dark followed by 4 h of light can induce extensive programmed cell death in the small somatic-like cells of a *gls/regA* mutant (dmAMN) compared to the somatic cells of a *V. carteri* wild-type strain (Eve) [28]. The dmAMN mutant was isolated in our laboratory from a spontaneous somatic regenerator mutant (UTEX1877) that has a 365-bp insertion in the exon 6 of *regA*, resulting in a truncated non-functional RegA protein (electronic supplementary material, figure S3) [28]. To confirm that the dmAMN's increased sensitivity to stress is specifically due to the lack of a functional RegA protein (and not to the *gls* or other potential mutations), we subjected the parental somatic regenerator UTEX1877 strain to the same conditions, and evaluated the sensitivity to stress of its somatic-like cells in juvenile individuals, before they regenerate. Both 35 h of dark and 31 h of dark followed by 4 h of light induced extensive death in the somatic-like cells of the mutant, but not in the wild-type strain (figure 2*a*; electronic supplementary material, figure S4). Specifically, all observed mutant colonies showed dead cells, while only *ca* 50% of the wild-type individuals were estimated to have dead cells. Overall, chi-square tests of independence showed that there was a significant association between strains and resistance to stress, in both conditions ($\chi^2 = 10.15$, $p < 0.001438$; $\chi^2 = 32.40$, $p < 0.00001$) (electronic supplementary material, tables S2 and S3). Increased sensitivity to dark–light stress was also observed in several other *regA* mutants (regAMN, regA HB11A, regA 153-68; electronic supplementary material, table S1 and figure S4), suggesting that the inability to adjust to light availability can impose a substantial cell-level cost.

(b) Somatic regenerator mutants are also sensitive to heat stress

To test whether *regA* mutants are sensitive to other types of stress, we also exposed the wild-type and mutant strains to various combinations of heat stress and assessed cell death as soon as 2 h post-stress (figure 2; electronic supplementary material, figures S5–S7). Heat stress triggers the cellular production of damaging reactive oxygen species (ROS), and we previously found that a 2 h heat stress at 42.5°C induces a twofold increase in ROS, which results in the somatic cells expressing and releasing a sexual inducer that initiates sexual reproduction [31]. Higher levels of heat stress (i.e. temperature or duration) and ROS can induce cell cycle arrest, programmed cell death or necrosis, depending on cell type and developmental stage [32,33].

Figure 2*b* shows the response to a 2 h heat stress (at 44°C) of young individuals from the wild-type Eve strain, two somatic regenerators (regA 153-68 and regAMN) and the dmAMN gonidialess/regenerator mutant—all on distinct genetic backgrounds and with independent/distinct *regA* mutations (electronic supplementary material, table S1 and figure S3). All mutants shared a markedly different response to the heat stress relative to Eve. Specifically, while *ca* 50% of individuals in Eve cultures lacked dead cells, no colonies without dead cells were observed in the heat-stressed mutant cultures (figure 2*b*; electronic supplementary material, figure S5). Also, chi-square tests of independence showed that there was a significant association between strains and sensitivity to the heat stress (electronic supplementary material, tables S4–S8). For instance, the proportion of colonies with less than 10% dead cells was significantly different between Eve and each of the three mutants ($\chi^2 = 138.90$, $p < 0.00001$ for reg A165-38; $\chi^2 = 132.17$, $p < 0.00001$ for regAMN; $\chi^2 = 105$, $p < 0.0001$ for dmAMN).

In nature, *V. carteri* inhabits small vernal water bodies that dry up at the end of summer. The increase in water temperature initiates the sexual phase that produces resistant zygospores [34]. The temperature used in this study is similar to that reached during the induction of sexual reproduction in their natural environments [34]. However, this is likely not the only environmental change that will differentially affect *regA* mutants in nature. Damaging levels of ROS can be achieved through a series of combinations of abiotic factors, including nutrient, high light and heat stress [35]. Notably, we previously found that the twofold increase in ROS level required for sexual induction can also be achieved by a lower heat stress (2 h at 40°C) when additional ROS are produced through mitochondria [31]. A similar effect could be achieved when photosynthetic activities are affected as during high light exposure and nutrient limitation. Overall, our findings that *regA* mutants are more sensitive to stress argue that the lack of a functional *regA* could impact the survival of regenerator mutants at the end of summer, which will affect their representation in the subsequent year's population.

4. Discussion

Understanding how cooperation evolves and is maintained in the face of cheaters is an intriguing and often controversial topic. Most studies on cheating and its suppression have been focused on eusocial insects (with respect to the reproductive altruism expressed by workers; [23]), bacteria (especially regarding the production of public goods; e.g. [36]) and social amoebae (during the aggregative formation of the multicellular slug and the differentiation of non-reproductive stalk cells; e.g. [37]). Our study investigated cheating and its impact in the simple clonal multicellular species, *V. carteri*, in the context of the mechanisms that can stabilize reproductive altruism during the early evolution of clonal multicellularity.

Generally, the key mechanism that can both favour and maintain cooperative and altruistic behaviours is considered to be kin selection [6,38]. However, since cheaters can still occur even in systems with high relatedness levels, alternative/additional evolutionary mechanisms have been proposed to stabilize/enforce cooperation and suppress or police/

punish cheaters (e.g. [39,40]). Among these mechanisms, pleiotropy has gained interest as a potentially important means to stabilize cooperation [41–43].

Pleiotropy refers to a gene that affects more than one trait [44]. Commonly, pleiotropy is expected to constrain evolution because adaptive changes in one trait might be limited by the potentially negative effects on the other traits associated with the same gene. Yet, such negative associations have been suggested to stabilize cooperation in social amoebae and bacteria (e.g. [41,45]) as by linking costly cooperation with a trait that confers a personal benefit, the loss of the cooperative trait will elicit a personal cost. For instance, in the social amoeba *Dictyostelium discoideum*, cheaters that avoid the sterile stalk fate are negatively affected in other traits (e.g. adhesion, ability to form spores) also linked to the cooperation genes ([41] and references therein). Nevertheless, the significance of pleiotropy to the evolution of cooperation has recently become an issue for debate [46,47]. Specifically, it has been argued that pleiotropy might be important to stabilizing cooperation only in limited instances and contexts and that, in fact, cooperation might select for pleiotropy [46].

Our data provide additional evidence for the role of pleiotropy in limiting cheating in a very different system—i.e. a clonal multicellular species. The fact that in *V. carteri*, RegA not only establishes somatic cell fate but also confers resistance to stress indicates that the *regA* gene has antagonistic pleiotropic effects on cell fitness. Thus, by expressing *regA*—and the altruistic behaviour, somatic cells do pay a cost in terms of their own reproduction but gain a personal benefit in terms of increased resistance to stress. At the same time, by avoiding the somatic fate cheaters gain a benefit in reproduction, but incur a *personal cost* in their ability to withstand environmental stress.

We suggest that this personal cost associated with the pleiotropic effects of *regA* contributed to the evolution of soma in the lineage leading to *V. carteri*. Consistent with this suggestion, the closest homologue of *regA* in the single-celled *Chlamydomonas reinhardtii* (known as *RLS1*) [16,48] is a life-history trade-off gene with antagonistic effects on reproduction and survival in limiting environments (i.e. suppresses reproduction to increase survival). Furthermore, although *RLS1* mutants have an immediate reproductive advantage, they pay a cost in long-term survival [49]. These findings together with the fact that orthologues of *regA* have been identified in species without somatic cells [50] (e.g. *Eudorina*; figure 1*a*) suggest that the antagonistic pleiotropic effects of *regA* preceded the evolution of reproductive altruism rather than being later linked to altruism.

We have previously proposed that the evolution of reproductive altruism/soma involved the co-option of pre-existing life-history trade-off genes [16]. Here, we argue that since by definition such genes have antagonistic effects on fitness, co-opting trade-off genes into somatic cell differentiation pathways can also contribute to stabilizing the new phenotype by providing a built-in safety system against cheaters, as mutations restoring reproductive potential will also inflict a survival cost. Such systems are likely still in place in other multicellular lineages and might act as first lines of defence against cheaters. Notably, the increased proliferation of cancer cells (somatic cheaters) was shown to be linked to increased sensitivity to nutrient stress due to their failure to trade-off cell proliferation for maintenance in stressful environments [51,52]. Since such trade-offs are common in single-celled organisms (e.g. [53–56]), this pleiotropic link is likely inherited from the unicellular ancestors of animals and might be involved in the purging of most pre-cancerous cells. Overall, we suggest that the *co-option of pre-existing* genes with pleiotropic effects, such as life-history trade-off genes, into cooperative traits has facilitated the early evolution of clonal multicellularity in other lineages.

Data accessibility. The data are provided in the electronic supplementary material [57].

Authors' contributions. M.E.C.-P.: conceptualization, data curation, formal analysis, investigation, methodology, visualization and writing—review and editing; S.G.K.: conceptualization, data curation, formal analysis, investigation, methodology, visualization and writing—review and editing; A.R.-G.: conceptualization, investigation, methodology, visualization and writing—review and editing; A.R.-P.: conceptualization, data curation, formal analysis, investigation, methodology, software, supervision, visualization and writing—review and editing; A.M.N.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, writing—original draft and writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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