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Evaluating the role of reproductive constraints in ant social evolution

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The reproductive division of labour is a key feature of eusociality in ants, where queen and worker castes show dramatic differences in the development of their reproductive organs. To understand the developmental and genetic basis underlying this division of labour, we performed a molecular analysis of ovary function and germ cell development in queens and workers. We show that the processes of ovarian development in queens have been highly conserved relative to the fruitfly *Drosophila melanogaster*. We also identify specific steps during oogenesis and embryogenesis in which ovarian and germ cell development have been evolutionarily modified in the workers. These modifications, which we call 'reproductive constraints', are often assumed to represent neutral degenerations that are a consequence of social evolutionary forces. Based on our developmental and functional analysis of these constraints, however, we propose and discuss the alternative hypothesis that reproductive constraints represent adaptive proximate mechanisms or traits for maintaining social harmony in ants. We apply a multi-level selection framework to help understand the role of these constraints in ant social evolution. A complete understanding of how cooperation, conflict and developmental systems evolve in social groups requires a 'socio-evo-devo' approach that integrates social evolutionary and developmental biology.

Keywords: ants; reproduction; development; germ cells; evo-devo; social evolution

1. INTRODUCTION

Evolutionary developmental biology (evo-devo) is bringing development into the modern synthesis and the mainstream of evolutionary theory (Hall 1999; Wilkins 2002; Eberhard 2003; Carroll *et al.* 2004). Evo-devo, however, has yet to be integrated with the field of social evolution, which was a critical theme arising from the modern synthesis. This is in spite of the fact that the social environment of individuals in a population can influence their development, and in turn development can feed back to influence their social environment (Toth & Robinson 2007; Smith *et al.* 2008). Thus, a 'socio-evo-devo' approach is needed for a complete understanding of how cooperation, conflict and developmental systems evolve in social groups (Toth & Robinson 2007). To understand the crosstalk between social evolutionary forces (ultimate mechanisms) and developmental processes (proximate mechanisms), this socio-evo-devo approach must integrate the concepts and approaches of both social evolutionary and developmental biology.

Here, we focus on a key feature of eusociality in ants—the reproductive division of labour between queens and workers—where queens perform most of the reproduction and workers perform the rest of the tasks such as foraging and brood rearing (Hölldobler & Wilson 1990, 2008). Workers in the majority of ant species have retained their ovaries, but do not have the same reproductive capacity as the queen because there exist several constraints on worker reproduction (Hölldobler & Wilson 1990, 2008). We call these constraints 'reproductive constraints', and define them as any process that quantitatively or qualitatively reduces the ability of workers to reproduce relative to the queen. Reproductive constraints can act through development or behaviour. Many behavioural reproductive constraints exist, which include policing (where workers aggress reproducing workers or kill each other's eggs) or self-restraint (where workers would refrain from reproducing). Here, we focus only on reproductive constraints that involve changes in the development of reproductive organs in worker ants. We consider constraints to be different from one another if they originate from the modification of distinct developmental processes in the workers. Based on this criterion, we identified five types of reproductive constraints, which we call RC1–RC5: RC1 acts through the mislocalization of maternal determinants (mRNAs and proteins) during late oogenesis and leads to embryonic failure of worker offspring (Khila & Abouheif 2008); RC2 affects the quantitative activity of the ovaries in adult workers; RC3 refers to loss of the sperm storage organ (spermatheca); RC4 refers to the reduction of ovariole number in the workers; and finally, RC5 refers to the complete loss of worker reproductive organs. Our general goal is to analyse the molecular and developmental basis of these constraints on worker reproduction and attempt to place them in the context of social evolutionary theory.

Inclusive fitness or kin selection theory has been key to understanding the conditions under which natural selection will favour cooperation within a group of

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relatives. It can be summed in a mathematical expression called Hamilton's (1964*a*,*b*) rule:

$$rb \geq c,$$

where r is the genetic relatedness between individuals in a population, b is the benefit, in terms of extra offspring that the beneficiary gains and c is the cost in terms of offspring loss that the altruist suffers. Thus, if Hamilton's inequality is satisfied, then the gene for altruism will spread throughout a group of individuals. In general, Hamilton's rule highlights both the genetic factors (as they affect relatedness) and ecological factors (as they affect costs and benefits) that promote cooperation and conflict in social groups (Bourke & Franks 1995). Although kin selection theory has long dominated the field of social evolution, it remains animated with controversy—most often ignited by the ideas and semantic confusion surrounding the alternative theoretical framework of group selection (Wilson & Hölldobler 2005; Fletcher *et al.* 2006; Foster *et al.* 2006*a*,*b*; Lehmann *et al.* 2007; West *et al.* 2007, 2008; Wilson & Wilson 2007; Kohn 2008; Wilson 2008*a*,*b*; Pennisi 2009). This is in spite of the fact that theorists in social evolution have for a long time agreed that, if certain conditions are satisfied, the theories of multi-level selection and kin selection are mathematically equivalent to one another and give the same predictions (Bourke & Franks 1995; Lehmann *et al.* 2007). These conditions are satisfied as long as in multi-level selection theory a 'within-group' and a 'between-group' component of selection are specified and individuals within these groups form a physical group of relatives, which is the case for social insects (Bourke & Franks 1995; Korb & Heinze 2004). Under this multi-level selection perspective, a gene promoting altruism will spread if the within-group conflict is weaker than between-group selection (Bourke & Franks 1995). Although there is still debate about which framework is the most useful (West *et al.* 2007, 2008; Wilson 2008*a*,*b*), we discuss the role of reproductive constraints using a multi-level selection framework (Korb & Heinze 2004).

However, several theoretical models of social evolution (Bourke 1999; Jeon & Choe 2003; Wenseleers *et al.* 2004; Reeve & Holldobler 2007; Gardner & Grafen 2009) implicitly or explicitly assume that reproductive constraints that affect development are neutral degenerations of worker ovaries in response to either kin or multi-level selection. Bourke (1999), for example, hypothesizes that as the size of a colony increases, it reduces the probability that any particular worker in the colony can replace the queen. This in turn increases selection for workers to mutually inhibit each other's reproduction through worker policing. This leads to the eventual decay or loss of worker reproductive potential, which allows for greater morphological differences to evolve between the queen and the workers.

In this article we propose an alternative hypothesis for the role of reproductive constraints—that they are adaptive proximate mechanisms or traits that reinforce social harmony in ants. To support our hypothesis, we first show that the processes of ovarian development and function in queens have been highly conserved relative to the fruitfly *Drosophila melanogaster*. We then identify the likely developmental genetic basis of several reproductive constraints, which occur at defined steps during development. Based on these results, we argue that the development of worker reproductive organs has been modified through a specific and narrow range of developmental parameters, which may not be expected if ovary modifications in the workers were a result of neutral decay mechanisms. Given this narrow range of developmental parameters, we also propose that worker ovaries may have been selectively modified to fulfil a range of alternative functions, including trophic egg production (Crespi 1992) and signalling physiological instructive cues to the behavioural response systems (Amdam *et al.* 2006).

2. MATERIAL AND METHODS

(a) *Preparation of whole reproductive organs*

Females were anaesthetized with carbon dioxide and immersed in PTW (1×phosphate buffered saline (PBS); 0.05% Tween-20). Reproductive organs can be recovered by pulling with forceps on the last tergite. After removing fat and unrelated tissues, images of reproductive organs were captured using a Zeiss scope and the AxioVision software.

(b) *Cloning and* in situ *hybridization of* Aphaenogaster rudis tor

Total RNA was extracted from queen ovaries, and used as template in a first-strand complementary DNA synthesis reaction (Invitrogen). This first-strand cDNA pool was used as a PCR template to amplify a 1600 base pairs (bp) *tor* fragment. The following forward and reverse *tor* primers were synthesized based on a *tor* sequence alignment from closely related insects: forward: 5′-TAGCCGAGTTTATGGAACATTG CGA-3′ and reverse: 5′-TGCGGTATAACTTGTA GCCAAGTGTTT-3′. Sequence alignment using ClustalW showed high levels of amino acid similarity between *Aphaenogaster rudis* and honeybee TOR protein, indicating that the cloned fragment represents *A. rudis tor* locus. *Aphaenogaster rudis tor* sequence can be retrieved in GenBank under the following accession number: GQ892187. *In situ* hybridization was conducted following the protocol in Khila & Abouheif (2009).

(c) *Ovary and embryo dissection and fixation*

Ovaries were dissected in PTW (1×PBS; 0.1% Tween-20) and kept on ice during the dissection process. The peritoneal sheet covering each ovariole was removed using fine forceps. The ovarioles were fixed in 4 per cent paraformaldehyde (200 µl) supplemented with 10 per cent DMSO (20 µl) and heptane (600 µl) for 20 min at room temperature. The fixed ovarioles were then washed three times in PBT (1 × PBS; 0.3% Triton X-100) and processed for subsequent staining. When processed for actin or microtubule staining, tissue should not be treated with methanol at any stage of the fixation process.

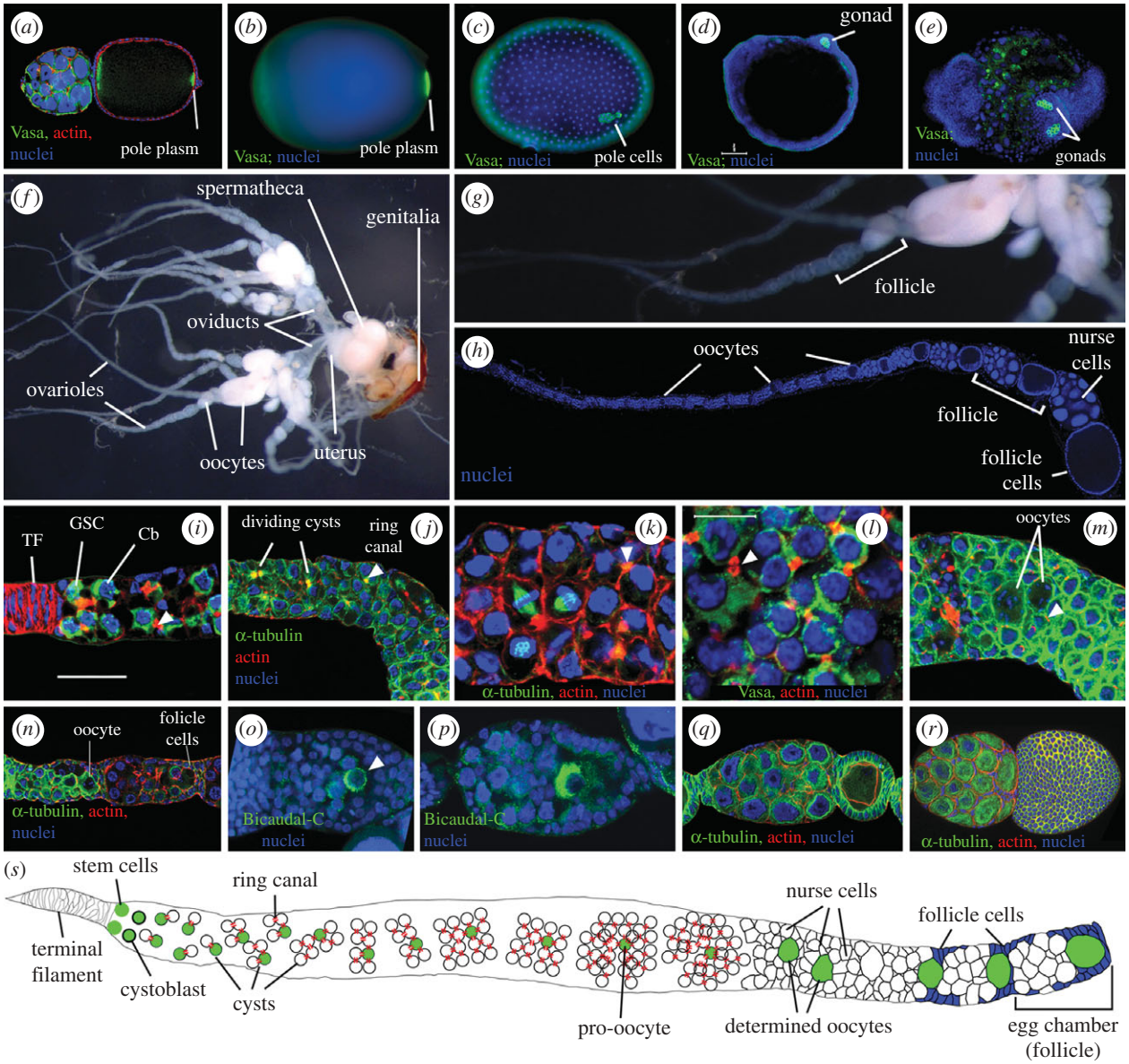

Figure 1. Germ cell development and oogenesis in ants. (*a–e*) Specification of the germ cells and formation of embryonic gonads. (*a*) The pole plasm, a specialized cytoplasm also known as germ plasm, is assembled in the posterior pole of the oocyte through the localization of multiple maternal determinants (here Vasa) to the posterior cortex. (*b*) The components of the germ plasm are inherited by the early embryo and incorporated in a cluster of cells, which thereby acquire a pole cell fate, at the cellularized blastoderm stage of embryogenesis. (*c*) Pole cells later on form the gonads (*d*), which split in two clusters (*e*) that later will give rise to either ovaries (females) or testes (males). (*f*) Typical reproductive organs of a queen of ants (here *Acromyrmex*). Queen reproductive organs contain two ovaries, each formed by several ovarioles connected to the uterus by oviducts. Note the presence of a prominent spermatheca, which is usually swollen in mated queens. (*g*,*h*) Focus on one ovariole captured by light microscopy (*g*) or stained with DAPI to reveal nuclei (*h*), showing follicles that grow in size as they approach the oviduct. As indicated, each follicle is formed by the oocyte and its cluster of nurse cells (both of germline origin) in addition to a layer of somatic follicle cells surrounding the oocyte. (*i*) At least two germline stem cells (GSC) can be found at the tip of the ovariole in close contact with the cells of the terminal filament (TF), in addition to the cystoblasts (Cb), which represent the immediate progeny of the GSC. Both GSC and Cb express high levels of the germline-specific protein Vasa (green colour). Note that the dividing Cb are connected with ring canals as revealed by actin staining in red colour (arrowhead); scale bar, 20 μm. (*j*,*k*) The cysts continue their incomplete divisions and the resulting daughter cells remain connected by microtubule (green colour) and cytoskeleton (red colour) material (arrowheads). (*l*) The cells forming the cysts are still indistinguishable from each other and continue expressing Vasa protein maintaining their germline identity; scale bar, 10 μm. (*m*) Two adjacent cysts where now one cell in each of the cysts appears larger than the rest and its nucleus shows signs that it has entered meiosis and therefore it has acquired oocyte fate. (*n*) The oocyte is now easily visible and has been encapsulated in a layer of follicle cells. (*o*) Initially, several cells of the cyst express the oocyte-determining marker Bicaudal-C (green colour), but the cell located posteriorly (arrowhead) express higher levels of the protein. (*p*) At this stage, only the oocyte expresses Bicaudal-C while the other cells of the cyst no longer express the protein. These cells have now adopted a nurse cell fate. Note the Bicaudal-C is localized in the anterior of the oocyte, indicating first signs of oocyte polarity. (*q*,*r*) The follicles are now growing in size, where the nurse cells actively synthesize maternal determinants that they provide to the oocyte. (*s*) A simplified schematic diagram of oogenesis (from *i* to *r*).

Embryos should be placed in an Eppendorf 1.5 ml tube in PBT, boiled for 30 s and then quickly placed on ice. The chorion and vitelline membrane can then be removed manually using fine forceps. Embryos can be then fixed in 4 per cent formaldehyde and heptane for 20 min, and then washed three times in PBT.

(d) *Antibody staining*

Cytoskeleton F-actin filaments were stained using rhodamin–phalloidin and nuclei using DAPI (Invitrogen). Vasa protein was revealed using a rabbit anti-Vasa antibody raised against *Drosophila* Vasa protein (Styhler *et al*. 1998) and Bicaudal-C (Bic-C) protein using a rabbit anti-Bic-C (Chicoine *et al*. 2007). Alpha-tubulin microtubule filaments were stained using the rat YL anti-alpha-tubulin antibody raised against yeast alpha-tubulin (Kilmartin *et al*. 1982). Fixed ovarioles or embryos were permeabilized in PBT (1 × PBS; 1% Triton X-100) for 1 h at room temperature. The permeabilization is followed by an antigen-blocking step in PAT (1 × PBS; 1% Triton X-100 and 1% bovin serum albumin) for 1 h at room temperature. The ovarioles/embryos were then incubated with a rabbit anti-Vasa antibody or YL rat anti-alpha-tubulin or both at the same time, at a 1 : 100 dilution in PTW overnight at 4°C. The ovarioles/embryos were washed off the excess antibody five times for 10 min at room temperature in PBT (1×PBS; 0.3% Triton X-100), and then blocked again in PAT for 1 h. A secondary anti-rabbit antibody (Jackson's labs) was used to detect the rabbit anti-Vasa antibody, while a goat anti-rat was used to detect the rat anti-tubulin, both at a 1 : 300 dilution in PTW. The ovarioles/embryos were incubated with the secondary antibody for 2 h at room temperature in PTW. DAPI and phalloidin were added at the same time as the secondary antibody. Ovarioles/embryos were finally washed five times for 10 min in PTW, then in increasing concentrations of glycerol in PBS and mounted in glycerol/1 × PBS (80/20%). Images were captured with an LSM 510 inverted confocal microscope and processed using Adobe Photoshop.

3. RESULTS

(a) *Conserved processes of germline and ovary development between ants and flies*

Insect reproductive organs form from two distinct cell populations (Hartenstein 1993). The first represents the germ cells, which contribute to the formation of the gonads and ultimately adult ovaries (figure 1*a–e*); the second is the genital disc, which gives rise to various somatic structures of the female reproductive organs, including the oviducts, uterus, spermatheca and other accessory structures (figure 1*f*). Germ cells represent one of the earliest specialized tissues to form during the development of many insects, including multiple ant species (Spradling 1993; Khila & Abouheif 2008). These cells are of special interest since they carry the heritable information, and thus play a unique role in evolution (Extavour & Akam 2003; Johnson *et al*. 2003). Pole cells, the precursors of germ cells, are specified, in ants as in flies, through the process of preformation (Khila & Abouheif 2008) (figures 1*a–e* and 4[a1–b2]). In this process, germline specifying gene products, including Vasa protein, are provided by the nurse cells to the developing oocytes where they become localized to the posterior pole (figure 1*a*). These products form the pole plasm, which is inherited by early embryos (figure 1*b*) and subsequently incorporated into a cluster of cells located at the posterior pole (figure 1*c*), thereby acquiring pole cell fate. Later on during embryonic development, pole cells migrate to the gonads (figure 1*d*), and in association with somatic mesodermal cells, split in two groups and form a pair of gonads (figure 1*e*). The female gonads continue development during larval stages and give rise to the ovaries and their associated somatic cell types (peritoneal sheath, terminal filament cells, follicle cells and other cell types; figure 1*f–h*). The remaining somatic structures of the female reproductive organs develop independently from the genital disc: a somatic cell population that is specified during later stages of embryonic development (Hartenstein 1993; Sanchez & Guerrero 2001). Only during pupal development (metamorphosis) do the ovaries fuse together with the products of the genital disc to form the complete reproductive organ (Hartenstein 1993). In adult reproductive organs, each ovary is composed of multiple string-like units known as ovarioles (figure 1*f*,*s*). The string-like appearance of each ovariole (figure 1*g*,*h*,*s*) reflects the continuous and independent production of ovarian follicles, which are pushed as they grow from the anterior (stem cell region) towards the posterior (oviduct region) (Spradling 1993; Riechmann & Ephrussi 2001). The steps of oocyte production, from the division of stem cells to the maturation of the oocyte (figure 1*i–s*), are tightly controlled through precise cellular and genetic processes. To make our analysis of the effect of social selective pressures on ovary development and function accessible, we describe the process of ant oogenesis in three major steps.

(i) *Step 1: stem cell self-renewal and differentiation*

Germline stem cells (GSCs; figure 1*i*,*s*) are essential for ovariole function as they ensure continuous oocyte production (Wong *et al*. 2005). In flies, two to three GSCs can be identified at the anterior tip of the ovariole, in tight physical association with their niche composed of cap cells and terminal filament cells, both of somatic origin (Lin *et al*. 1994; de Cuevas *et al*. 1997; Song *et al*. 2004). GSCs in ants can only be inferred from their position (figure 1*i*,*s*), and specific molecular markers are yet to be developed (Khila & Abouheif 2008). Queen GSCs express high levels of Vasa protein (green colour in figure 1*i*), suggesting that Vasa may be necessary for the self-renewal and differentiation processes in ants. This is consistent with Vasa expression and function of fly GSCs. Actin cytoskeleton and alpha-tubulin staining reveal that in ants, GSCs divide asymmetrically resulting in two daughter cells with opposite developmental fates (figure 1*i–k*,*s*). The first daughter cell remains attached to the niche to ensure the renewal of the GSC and the continuity of the process (figure 1*i*,*s*). The second acquires a cystoblast identity (figure 1*i*,*s*)

and activates differentiation programmes, moves away from the niche and engages in successive incomplete divisions to become a cyst (figure 1*i–n*,*s*) (de Cuevas *et al.* 1996). This distinct fate of GSC progeny in flies is instructed by molecular signals emanating from the neighbouring niche cells (Wong *et al.* 2005). This suggests that similar signals may be responsible for GSC self-renewal and differentiation in ants. Each cystoblast undergoes five incomplete divisions in ants, resulting in 32 interconnected cells that form the cyst, whereas in *Drosophila* only four divisions occur, resulting in 16-cell cysts (van Eeden & St Johnston 1999).

(ii) *Step 2: determination of oocyte fate and polarity*

At each division of the cystoblast, the resulting daughter cells remain connected by cytoplasmic bridges called ring canals, which ensure communication between the cells forming the cyst (figure 1*i–m*,*s*) (van Eeden & St Johnston 1999; Huynh & St Johnston 2004). In ants, four to five cells located at the posterior of the cyst accumulate Bic-C protein (figure 1*o*). Shortly after, Bic-C now accumulates only in the cell located most posteriorly in the cyst (figure 1*p*), indicating that this cell has acquired the oocyte fate, consistent with this process in *Drosophila* (van Eeden & St Johnston 1999; Riechmann & Ephrussi 2001; Huynh & St Johnston 2004). The remaining cells of the cyst now exhibit large nuclei, indicating that these cells have adopted a nurse cell fate (figure 1*p–s*). In both ants and flies, only one cell is chosen as an oocyte and accumulates products of polarity genes (in addition to other cell cycle components) at its anterior pole (Huynh & St Johnston 2004). This cell enters meiosis, whereas the remaining 31 cells engage in multiple chromosomal endoreplications and become polyploid nurse cells (figure 1*p–s*). This results in the meroistic polytrophic mode of oogenesis, where a cluster of nurse cells assists each oocyte (figure 1*q*,*s*). The oocyte fate, after being specified, has to be maintained in order for oogenesis to progress (Cox *et al.* 2001; Huynh & St Johnston 2004). In ants, during the encapsulation of the cyst by follicle cells (figure 1*o*), four neighbouring cells in the cyst show a perinuclear accumulation of the Bic-C protein. However, the cell located most posteriorly in the cyst accumulates higher amounts of the Bic-C protein, indicating that this cell has been defined as an oocyte (arrowhead in figure 1*o*). Shortly thereafter, only this cell shows Bic-C accumulation, with a clear translocation of the protein to the anterior pole suggesting that this oocyte is already polarized at such an early stage. This indicates that the processes of oocyte determination and polarity establishment are highly conserved between ants and *Drosophila*.

(iii) *Step 3: patterning of the oocyte*

The active molecular crosstalk between the oocyte and the surrounding layer of somatic follicle cells is essential for establishing the polarity of the egg chamber. The localization of three main molecules in *Drosophila*, Gurken protein, *bicoid* mRNA and Oskar protein, initiates the major events leading to the patterning of the oocyte in preparation for early embryogenesis (Steinhauer & Kalderon 2006). The patterning of the posterior compartment in ants involves the localization of conserved maternal determinants, including Vasa protein and *nanos* mRNA (Khila & Abouheif 2008), and the assembly of the pole plasm in the posterior pole (figure 1*a*). These maternal determinants are synthesized by the nurse cells and transported to the oocyte through microtubule cytoskeleton to their respective locations (figure 1*n*,*q–r*). How the other compartments are established in ant oocytes remains unknown.

(b) *Reproductive function of worker ovaries is blocked or reduced at specific steps during oogenesis*

Queen and worker castes represent alternative phenotypes that are in general determined environmentally from the same genome (Wheeler 1917; Wheeler & Nijhout 1981; Nijhout & Wheeler 1982; Nijhout 1999). This caste polyphenism results in a dimorphic development between queen and worker reproductive organs. As a result, ovaries in the queen caste are fully developed (figure 2*a*), whereas ovaries in the workers have been modified during development and evolution, resulting in lower reproductive potential (figure 2*b*). An analysis of ovarian structure and function in the workers of two species, *Aphaenogaster treate* and *Lasius alienus*, shows that the reproductive function of worker ovaries is blocked or reduced through targeting specific and different steps of oogenesis. This raises the possibility that reproductive constraints represent adaptive proximate mechanisms that have evolved to reduce worker reproduction, and direct worker ovaries towards alternative functions, thereby reinforcing social harmony.

In the workers of *A. treate* and *L. alienus*, the cellular and structural organization of the ovarioles is qualitatively preserved (figures 1 and 2*b–f*). The GSCs are present and properly renewed, the cysts divide properly and the follicles contain one oocyte and the right number of nurse cells. This is likely to be a general feature in the workers of ants. In *A. treate*, however, worker ovaries are quantitatively constrained, thus providing an example of RC2. Our data suggest that ovarian activity has been targeted through reducing the rate of GSC division in the workers (step 1 of oogenesis). The length of the ovariole indicates the number of developing follicles, which directly reflects the rate of GSC division and cyst production (LaFever & Drummond-Barbosa 2005; Hsu & Drummond-Barbosa 2009). In the presence of the queen, worker ovarioles (figure 2*b*) are generally shorter than queen ovarioles (figure 2*a*), suggesting that the rate of GSC division and oocyte production is slower in the workers. However, some workers dramatically increase the activity of their ovarioles in the absence of the queen (figure 2*c*). The insulin pathway may mediate the differential control of ovarian activity between queens and workers, because in flies the rate of GSC division and cyst production is directly controlled through this pathway (LaFever & Drummond-Barbosa 2005; Hsu & Drummond-Barbosa 2009). Consistent

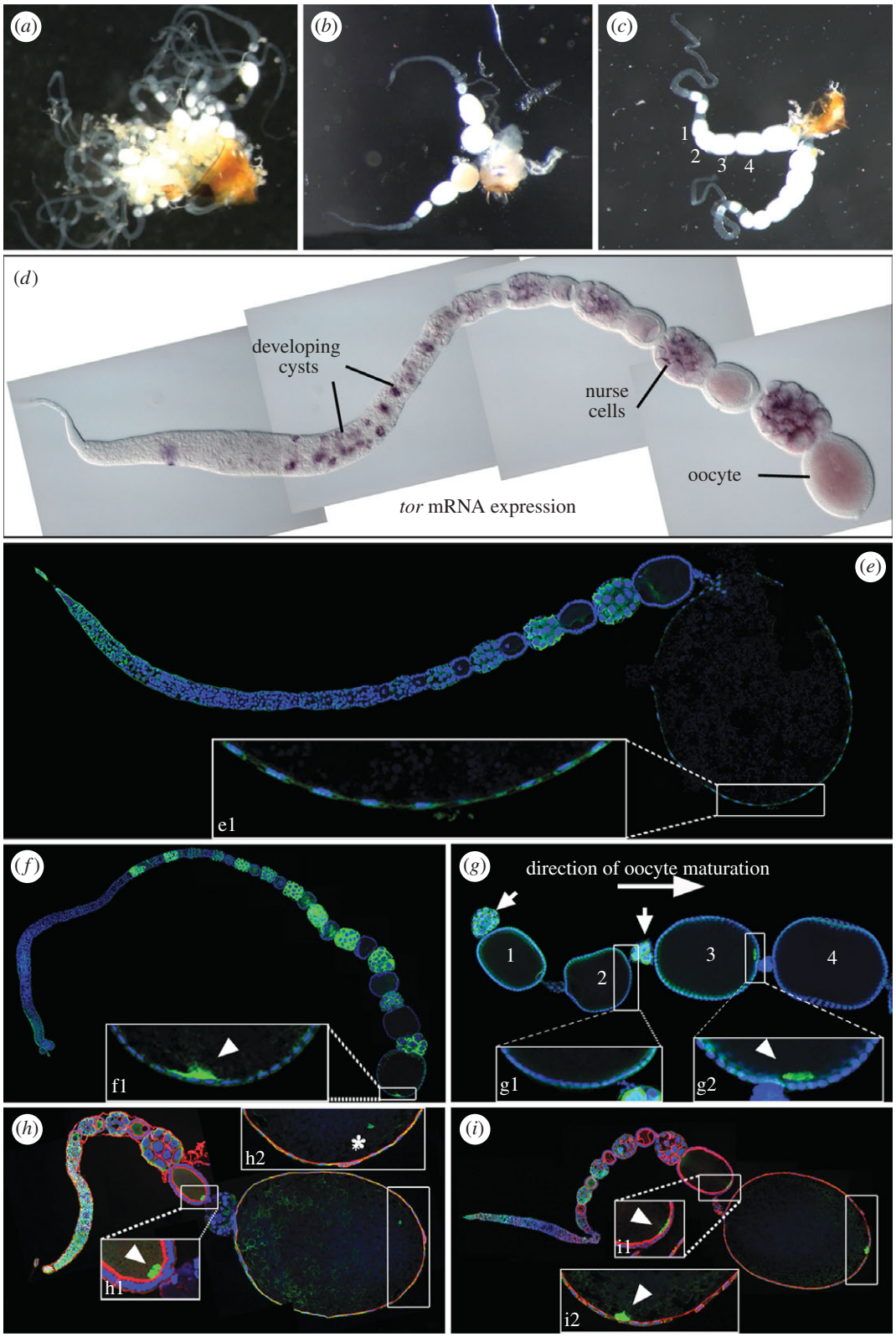

Figure 2. Functions of female reproductive organs in ants. (*a*) Reproductive organs of a queen of *A. rudis* showing the high number of ovarioles in the ovaries. (*b*) Reproductive organs of a worker, from a queened colony, where each ovary contains only one short ovariole. (*c*) Reproductive organs of a worker from a colony without a queen, showing that the ovarioles are now longer, which indicates much higher activity. (*d*) Ovariole from an *A. rudis* worker showing the expression of *tor* mRNA in the early developing oocytes and in the nurse cells. *tor* mRNA is transported from the nurse cells to the oocyte in older follicles. (*e*) Ovariole of a worker from a queened colony, stained for nuclei (blue colour) and Vasa protein (green colour). Note in the mature oocyte the absence of Vasa localization at the posterior pole (e1), indicating that this oocyte is trophic. (*f*,*g*) Same ovariole as in (*c*), of a worker from a colony without a queen, stained for nuclei (blue colour) and Vasa protein (green colour). In addition to the higher number of follicles, many oocytes now localize Vasa to the posterior pole (f1 and g2) while other do not (g1). This indicates that the same ovariole is producing both trophic and reproductive oocytes in the absence of the queen. (*h*,*i*) Ovarioles of *L. alienus* workers from a colony without a queen, stained for nuclei (blue colour), actin (red colour) and Vasa (green colour). Note that these workers make oocytes with mis-localized Vasa (h2) and others with correct Vasa localization (h1, i1 and i2).

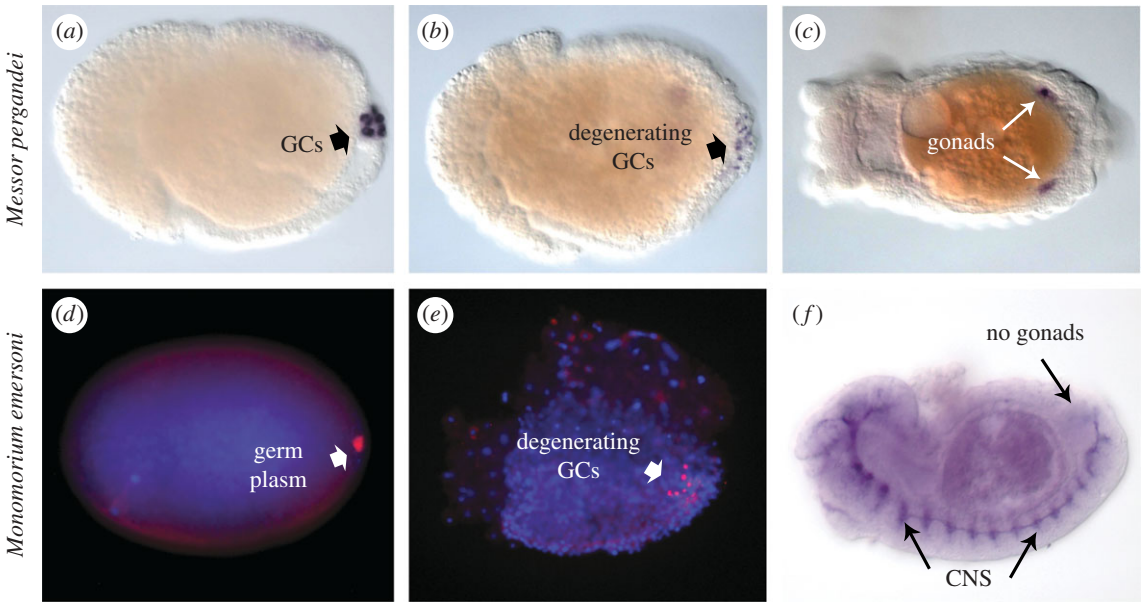

Figure 3. The developmental basis of germ cell reduction or loss in the workers. (*a–c*) Worker-destined embryos of the ant *M. pergandei* stained for *nanos* mRNA to reveal the germ cells (GCs). (*a*) Embryo at early gastrulation, a stage where the germ cells are still developing properly. (*b*) A slightly older embryo showing that the germ cells are now degenerating. (*c*) A late embryo showing the remainder of germ cells, which have populated the gonads. (*d–f*) Embryos of the ant *M. emersoni* stained for Vasa protein to reveal the germ cells. (*d*) Early embryo showing the proper formation of the germ plasm at the posterior pole. (*e*) Embryo at gastrulation showing that the germ cells are undergoing degeneration. (*f*) A late embryo showing that the gonads are absent, and that Vasa now accumulates in the central nervous system (CNS).

with this hypothesis, we found that the ovarioles of the ant *A. rudis* express mRNA of the protein kinase *tor* (*target of rapamycin*) throughout oogenesis (figure 2*d*). *tor* is a major component of the insulin signalling pathway (Fingar & Blenis 2004), and its expression represents a preliminary indication that this pathway is conserved and active in ants. Therefore, RC2 represents the ability to quantitatively regulate worker ovaries in response to their social environment.

This RC2 in *A. rudis* has evolved such that ovarian activity is partially maintained in the workers when the queen is present. This allows the production of trophic eggs in colonies with functional queens. Indeed, ovaries of these workers contain only oocytes that lack Vasa protein in their posterior pole (figure 2*e*,e1). We have previously established that oocytes that lack maternal determinant localization in the posterior pole will become trophic eggs (Khila & Abouheif 2008). In the absence of the queen, ovaries of some workers become much longer, indicating higher activity (figure 3*c*). These ovaries, in the absence of the queen, produce both trophic and reproductive oocytes. This is shown by the presence of oocytes both with (figure 2*f*,f1,*g*,g2) and without (figure 2*g*,g1) Vasa localization in the posterior pole (Khila & Abouheif 2008). Therefore, RC2 allows some workers to switch their ovarian activity towards reproduction, probably to produce one last cohort of males in the case where the queen is lost. In addition to RC2, workers in this species also produce oocytes with mis-localized maternal determinants (RC1), have lost the spermatheca (RC3; figure 2*b*,*c*) and have ovaries with a reduced number of ovarioles (RC4; figure 2*b*,*c*).

In *L. alienus*, worker ovaries are constrained through maternal determinant mis-localization providing an example of RC1 (figure 2*h–i*). Our data show that oogenesis in these workers has been targeted at late stages (step 3). The workers of *L. alienus* produce oocytes that either fail to localize Vasa (failed oocytes; figure 2*h*,h2) or show a correctly localized Vasa pattern (viable oocytes; figure 2*h*,h1, *i*,i1 and i2). Furthermore, even when Vasa shows a correct localization in young oocytes, this localization can be lost later during oogenesis. We found that in the same ovariole, young oocytes show correctly localized Vasa (figure 2*h*,h1), whereas older oocytes lose this localization (figure 2*h*,h2). Therefore, RC1 represents the mis-localization of maternal determinants, such as Vasa, which results in developmental failures of the future embryos, and thus, reduced reproduction (Khila & Abouheif 2008). The fact that we did not observe any trophic eggs in this species, together with the presence of RC1, raises the possibility that these ovaries may have been maintained for two possible functions: (i) the production of a last cohort of males if the queen is lost and/or (ii) may perform alternative functions other than trophic egg production. This second possibility is consistent with our previous finding in another ant, *Myrmica americana*, where the workers produce high percentages of failed oocytes and low percentages of trophic oocytes (Khila & Abouheif 2008). This conclusion is also consistent with the function of worker ovaries in the honeybees, where functionally sterile foragers will decide to collect either nectar or pollen based on the status of their reproductive organs (Amdam *et al.* 2006). It is possible that in the workers of ants, ovaries emanate or receive endocrine signals that regulate the worker's behavioural status.

(c) *Germ cells are reduced or lost during embryogenesis in the workers of many ant species*

The evolutionary reduction (RC4) or complete loss (RC5) of germ cells in the workers represents one of the most important aspects of the reproductive division of labour in social insects. In the majority of ants, workers develop reduced reproductive organs where parts of both somatic and germline structures have been removed. Only in a few genera (nine out of 283) have workers completely lost their reproductive organs. These are: *Solenopsis, Eciton, Hypoponera, Anochetus, Leptogenys, Monomorium, Tetramorium, Pheidole* and *Pheidologeton* (Oster & Wilson 1978; Hölldobler & Wilson 1990; Villet *et al.* 1991). Because germ cells in ants, as opposed to honeybees, are specified early during embryo development, we predicted that they would be reduced or eliminated at low cost during embryogenesis. To test this prediction, we analysed the development of germ cells in the embryos from colonies that produce only workers, using the two germline-specific markers *nanos* mRNA (figure 3*a–c*) and Vasa protein (figure 3*d–f*) (Styhler *et al.* 1998; Dearden 2006; Khila & Abouheif 2008). We conducted this analysis in *Messor pergandei*, a species where workers have reduced number of ovarioles, and *Monomorium emersoni*, a species where workers have completely lost their reproductive organs.

We found that during late oogenesis and early embryogenesis, the germ plasm is assembled normally in worker-destined embryos of both *M. pergandei* (figure 1*b*) and *M. emersoni* (figure 3*d*). This is consistent with the fact the molecules forming the germ plasm play multiple roles in embryo development (Thomson & Lasko 2005), and affecting them at this stage would have deleterious consequences not only on the germ cells but also on other somatic processes. By early gastrulation, the pole cells first form normally and are positioned at the posterior pole of the embryo (figure 3*a*). However, at late gastrulation, the cluster of germ cells as revealed by *nanos* (figure 3*b*) or Vasa (figure 3*e*) now shows a pattern that indicates their degeneration in both *M. pergandei* and *M. emersoni*. By the end of embryo development, *M. pergandei* embryos still maintain a fraction of their germ cells, which now populate the two gonads (figure 3*c*). This is not the case for *M. emersoni*, where late embryos do not show any Vasa expression in the gonads, but surprisingly show a pattern of Vasa expression that has been co-opted to the central nervous system. This is the first study, to our knowledge, showing a germline-specific gene, such as *vasa*, being co-opted on to the nervous system. Since all embryos examined come from colonies that produce only workers, these results provide an indication that both the reduction and complete loss of ovarioles in the workers occur during embryogenesis. We have uncovered this correlation in multiple ant species (data not shown) between the reduction of germ cells and that of the ovaries (*A. rudis*), and between the loss of germ cells and the loss of ovaries (*Tetramorium caespitum*, *Pheidole dentata*, *Pheidole morissi*). This suggests that embryos in these species may not be totipotent, and that caste determination may occur during embryogenesis rather than larval development. This may provide an efficient method for determining whether caste determination occurs during embryonic or larval development in other species. Reducing or eliminating germ cells at late gastrulation indicates that the mechanism responsible for RC4 and RC5 is highly selective.

4. DISCUSSION

Our results show that the process of oogenesis is highly conserved between ants and the fruitfly *Drosophila*, and that in the workers of different ant species this process is blocked or reduced at different and specific steps. The specificity through which worker ovaries are modified is such that these ovaries may still be able to perform alternative functions (nutritional and/or physiological). We therefore propose the hypothesis that reproductive constraints may not represent neutral degeneration of worker ovaries, but rather represent adaptive traits that block or reduce worker reproduction. We discuss each of the reproductive constraints (RC1–5) in the light of this hypothesis, and propose that their role in ant social evolution may be to reduce within-colony conflict and increase between-group competitiveness.

(a) *RC1: mis-localization of maternal determinants*

RC1 is the mis-localization of specialized mRNAs and proteins necessary for the proper patterning of the oocytes produced in worker ovaries (figures 2*h*,h2 and 4[a3]), which leads to severe defects during embryo development (Khila & Abouheif 2008). This constraint is ultimate because it occurs in the workers both in the presence and absence of the queen (Khila & Abouheif 2008). RC1 should be considered as a mechanism because it acts on a process that is continuous in time, i.e. oogenesis. The ovariole is a string-like structure where growing oocytes move from the anterior towards the posterior (figure 1*s*; Spradling 1993). As they move towards the posterior, each oocyte is directed towards a trophic or reproductive fate. We infer trophic oocytes to be those that do not localize maternal determinants, and reproductive oocytes to be those that do localize these molecules. Since RC1 affects maternal determinant localization it can only affect reproductive, but not trophic oocytes. The fraction of the reproductive oocytes affected by RC1 can vary between species. Therefore, RC1 blocks the ancestral function of worker ovaries by reducing or eliminating the workers' ability to produce non-fertilized male eggs, without preventing trophic egg production.

Therefore, we hypothesize that RC1 may not represent a neutral decay of the ovaries but rather is an adaptive developmental mechanism that reduces or eliminates worker reproduction, while restricting the ovaries to performing a nutritional function. Such a mechanism can only act within a narrow range of developmental parameters during late steps of oogenesis. If earlier steps of oogenesis, such as GSC division and cyst production, are blocked this would eliminate both reproduction and trophic functions of the ovary. Mutations in a variety of genes in the fly indicate that there exists a genetic potential for completely blocking oogenesis as early as GSC

self-renewal and differentiation (see Wong *et al.* (2005) for review). These include *vasa*, *nanos*, *bag-of-marbles*, *decapentaplegic* and other molecules necessary for GSC self-renewal, cystoblast differentiation or other cellular processes necessary for continuous follicle production (Styhler *et al.* 1998; Wang & Lin 2004; Szakmary *et al.* 2005; Wong *et al.* 2005). The specificity of RC1 at late steps of oogenesis, together with the fact that evolution has not acted upon the potential to block early steps, suggests that RC1 represents an adaptive developmental mechanism rather than a neutral degeneration of worker ovaries.

(b) *RC2: quantitative constraint on ovary activity*

A general feature of insect ovaries is their ability to respond to environmental changes, such as hormonal input and changes in nutrition, by changing their activity (Drummond-Barbosa & Spradling 2001). This ovarian plasticity may have been refined in the workers of ants through the evolution of specific response elements that allow them to respond in a specific manner and to specific social cues in their environment. Therefore, RC2 may represent an adaptive developmental mechanism that can be defined as the ability of workers to regulate their ovarian activity in response to chemical and behavioural interactions between individuals, such as policing, self-restraint, and pheromones. RC2 is not an ultimate reproductive constraint, because in particular social conditions, such as the absence of the queen, worker ovaries may increase their activity. The regulation of worker reproduction through RC2 has evolved such that in the presence of the queen it may be partial in some species and total in others. *Aphaenogaster treate* is a monogynous species with a relatively large colony size and a pronounced queen/worker caste dimorphism. Our results show that in this species, worker ovaries are still active in the presence of the queen, allowing for the production of trophic oocytes. In contrast, species such as *Harpegnathos saltator* or *Amblyopone pallipes* form small colonies where queen and worker castes are hardly recognizable (Peeters & Hölldobler 1995; Peeters *et al.* 2000). In this type of species, worker ovaries in the presence of the queen are inactive, representing a total blockage of oogenesis.

The molecular mechanism of RC2 is still unknown. However, the outcome of RC2 affects the length of the ovariole, which reflects the rate of GSC division and cyst production. This suggests that this constraint may be directly linked to step 1 of oogenesis, i.e. GSC division and cyst production. The insulin-signalling pathway may mediate ovarian response to RC2, since our results suggest that this pathway may be conserved and active in ant ovaries (figure 2*d*). Thus, it is possible that signals from the queen, either directly or indirectly, suppress this insulin-like peptide production in the workers. Furthermore, it has been shown in *Drosophila* that this pathway directly controls the rate of GSC division and cyst production (LaFever & Drummond-Barbosa 2005). Insulin-like peptides are produced by a specialized cell cluster in the brain and mediate a direct and positive long-range effect on ovarian activity (Cao & Brown 2001; Riehle *et al.* 2006). This possibility is further supported by transcriptomics data from the brain of a social wasp, where the brain of the queen expresses much higher levels of the insulin-like peptide2 than that of the workers (Toth *et al.* 2007).

(c) *RC3: loss of the spermatheca*

In most ants, the spermatheca (sperm storage organ) has been lost, resulting in workers that cannot store sperm or fertilize the eggs they produce. These workers, in theory, can still produce unfertilized eggs, which may potentially develop into males owing to the haplo-diploid sex determination that characterizes ants. We hypothesize that RC3 is an adaptive trait that has been maintained by selection to block the production of daughters, which represents half the reproductive potential. This hypothesis is supported by the specificity of the processes through which the spermatheca develops. The spermatheca, along with the other somatic structures of the female reproductive organs (genitalia, oviducts and uterus), develops from the genital disc, which is specified during late embryogenesis and patterned during larval development (Hartenstein 1993; Sanchez & Guerrero 2001). Since the cell population that gives rise to the spermatheca is part of the genital disc and not independent, it may be more probable for the whole genital disc to be removed through degeneration, than for a fraction of its cells, i.e. those of the spermatheca, to be singled out. However, the loss of the entire genital disc has been selected against because an alternative ovary function, i.e. trophic egg production, requires the oviducts, uterus and genitalia to connect the ovarioles (where trophic eggs are produced) to the exterior (where these eggs are consumed).

A comprehensive test of our hypothesis would require analysing RC3 at multiple biological levels, i.e. anatomical, embryological and developmental genetic, in a phylogenetic framework. With respect to the anatomical and embryological levels, further work is required to determine at what stage of development (embryonic or larval) and to what degree (complete or partial) the spermatheca is lost in ants. With respect to the genetic basis of spermatheca development, little is currently known, although an old study in *Drosophila* has reported that females mutant for the gene *lozenge*, involved in apoptosis regulation, lack spermathecae (Anderson 1945; Wildonger *et al.* 2005). These data, once collected from multiple species, can be contrasted against a null phylogenetic model based on neutral degeneration. If RC3 were the consequence of neutral degenerative processes, then we should expect a phylogenetic pattern where the frequency and degree of loss would be more similar between closely related ant lineages than between distantly related ones regardless of the selective pressures that may arise from their social structure, ecology or life history (Abouheif 2003).

(d) *RC4: reduction of the number of ovarioles*

In most ant species, the number of ovarioles has been dramatically reduced in the workers to as low as one ovariole per ovary (figure 4[d2]), while queens possess

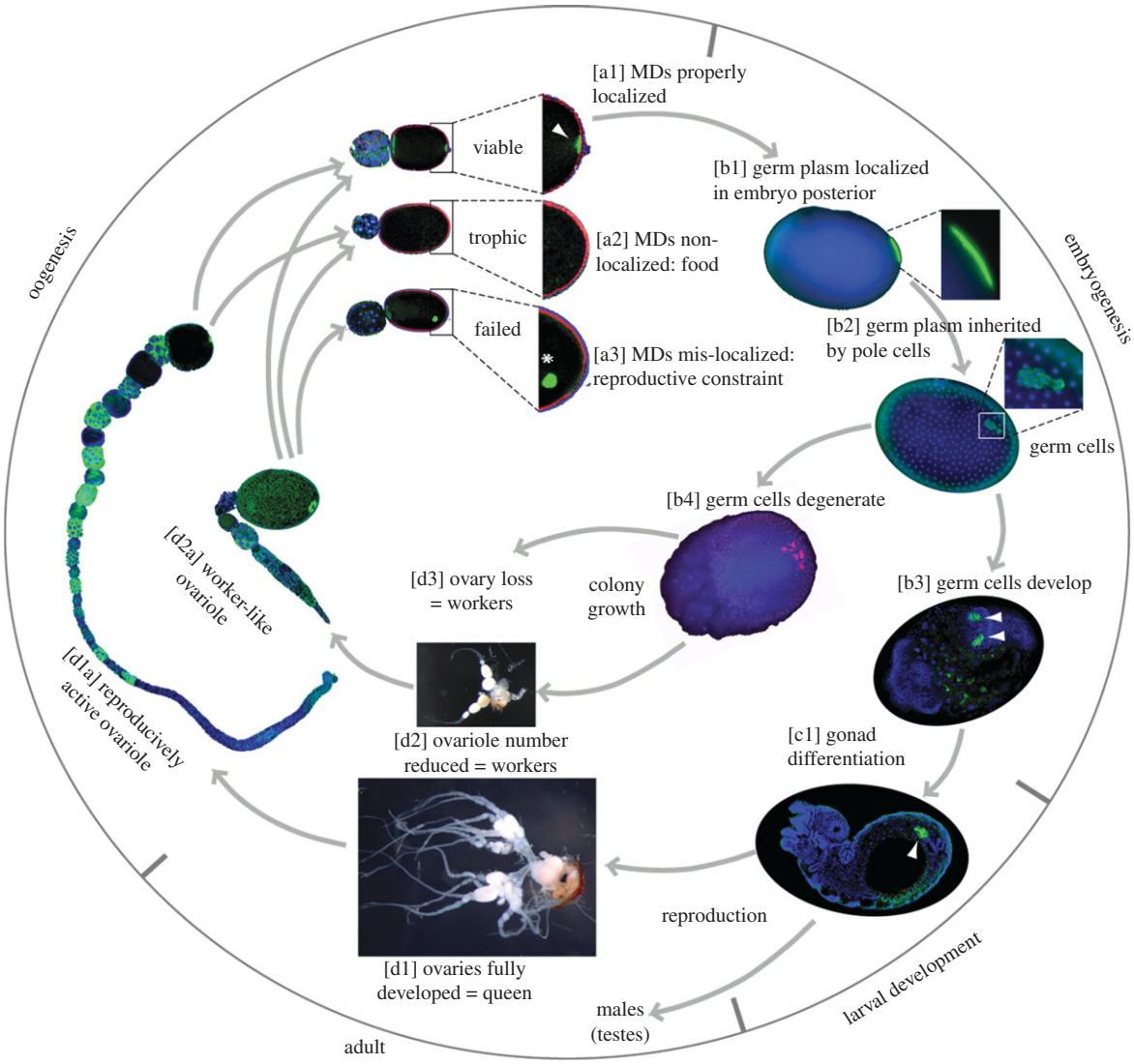

Figure 4. Summary of germ cell development and ovary function in ants. [a1–a3] Types of oocytes produced in ant ovaries. [a1] Viable with correct maternal determinant localization, [a2] trophic with no localization and [a3] failed where Vasa protein accumulates in the oocyte but its localization is not maintained at the posterior pole. Only oocytes with correct localization [a1] possess functional pole plasm and will undergo proper development. The pole plasm is inherited by the embryo [b1] and functions to specify the germ cells [b2]. Germ cells may develop fully [b3] and lead to the formation of complete gonads [b3 and c1] and therefore fully developed queen ovaries [d1]. Alternatively, germ cells may undergo degeneration during embryo development [b4] and lead to either reduced number of worker ovarioles [d2] or complete loss of worker ovarioles [d3]. Ovarioles of queens (or workers that are reproductively active) [d1a] are more active than normal worker ovarioles [d2a]. Queen ovarioles can produce both trophic and reproductive oocytes [a1–a2], whereas worker ovarioles are constrained at the molecular level and produce failed oocytes [a3] in addition to viable [a1] and trophic ones [a2]. Green, Vasa; blue, nuclei; red, actin.

several to several thousand ovarioles per ovary (figure 4[d1]). RC4 is a trait that is generally accompanied by the loss of the spermatheca (RC3). Embryonic pole cells, the precursors of the germline part of adult ovarioles, are specified in ants during oogenesis and early embryogenesis, representing one of the earliest tissues to be specified (figure 4[a1]–[b2]). We present data indicating that in ants the reduction of ovariole number may be caused by the degeneration of pole cells during the development of worker-destined embryos (figure 3). The developmental basis of RC4 is different in honeybees, where ovariole number is reduced in worker larvae rather than in embryos (Schmidt Capella & Hartfelder 2002; Reginato & Cruz-Landim 2003). This is consistent with the much later timing of germ cell specification in bees relative to ants (Dearden 2006).

We hypothesize that RC4 is an adaptive trait that has evolved to reduce worker reproduction without interfering with possible alternative functions of worker ovaries. Because the early stages of embryonic development are quite sensitive and involve simultaneous specification of various tissues using common gene batteries (Thomson & Lasko 2005), germ cell degeneration can only occur within a narrow range of developmental parameters. The process of germ cell specification and that of patterning the posterior of the embryo are tightly interdependent (Thomson & Lasko 2005). Many of the molecules that form the germ plasm, including Nanos and Vasa, are known to have pleiotropic functions in the development of both germ cells and posterior segments in addition to other key developmental processes. Our results indeed show that this reduction of germ cells

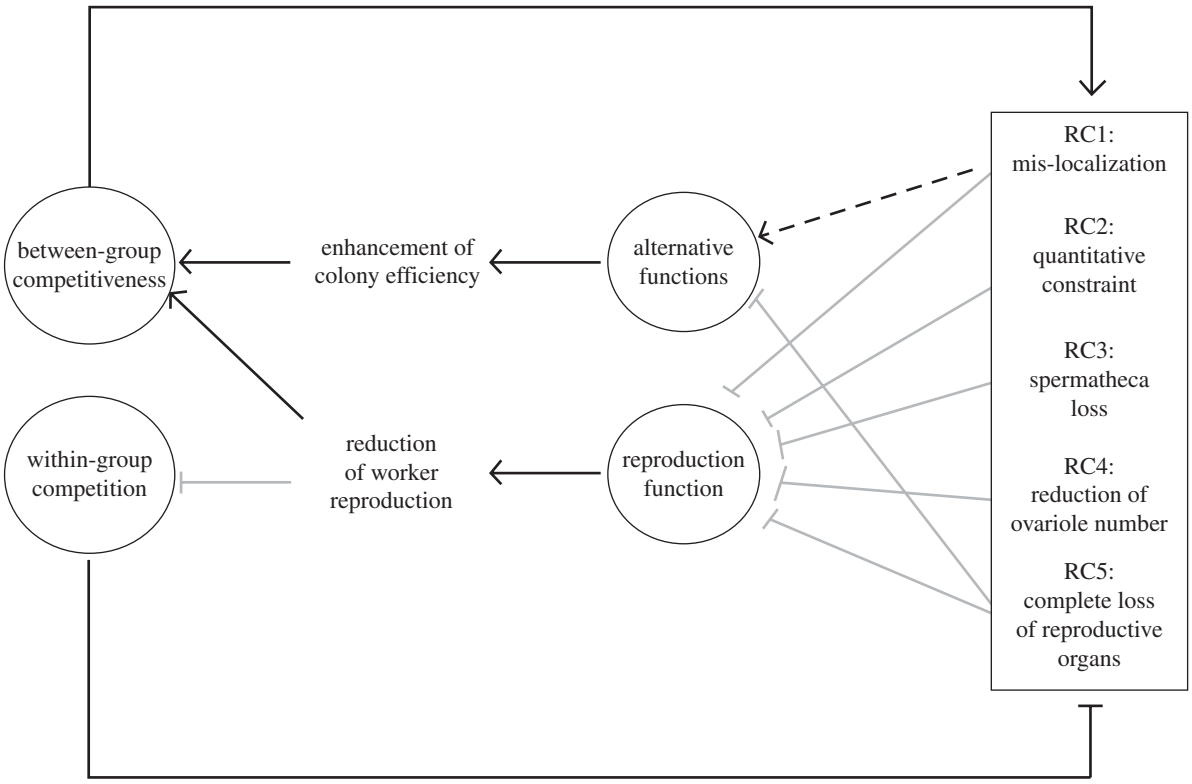

Figure 5. Crosstalk between reproductive constraints and multi-level selection solid lines with arrows indicate an increase, while truncated T-shaped lines indicate a decrease. The dashed line with arrow indicates that RC1 restricts ovaries to alternative functions. Reproductive constraints evolve as a consequence of social selective forces, but once they evolve, they positively feed back to decrease within-group competition and increase colony competitiveness.

occurs during gastrulation after embryonic posterior genes have fulfilled their patterning function (figure 3*b*,*e*). Furthermore, each ovariole is an independent functional unit in which GSCs are active and can produce their own oocytes independently from the other ovarioles (Spradling 1993). Therefore, reducing the number of ovarioles affects only the quantitative rather than the qualitative activity of the ovary. The probability for a neutral process to fully eliminate germ cells in the workers may be higher than to selectively target a fraction of them. Multiple specific mutations, known in flies, lead to the complete loss of germline cells (Gigliotti *et al.* 2000). Despite this genetic potential, workers in the majority of ants still maintain a fraction of their ovaries, suggesting that the reduction of ovariole number is targeted and positively selected for to fulfil their alternative functions.

RC4 is also widespread in bees and wasps, indicating that social selective forces will maintain ovaries in the workers as long as these ovaries have one or several alternative functions. In the honeybee, the division of foraging labour among functionally sterile workers is linked to the degree of their ovarian activity (Amdam *et al.* 2006; Smith *et al.* 2008). This is consistent with the fact that insect ovaries in general, in addition to gamete production, have a deep influence on the individual's physiology and behaviour through strong endocrine activity (Brogiolo *et al.* 2001; Ikeya *et al.* 2002; Davey 2007*a*,*b*; Broughton *et al.* 2008). This provides flexible systems where variation in maternal reproductive traits within the non-reproductive helpers will induce complex social behaviour among these helpers for more precise and efficient division of labour (Amdam *et al.* 2006; Tsuruda *et al.* 2008; Wang *et al.* 2009). This strong link between reproductive anatomy, physiology and behavioural response systems is likely to be a common feature of social insects in general. Altogether, these observations further support the hypothesis that RC4 may represent an adaptive trait that allows the maintenance of a fraction of worker ovaries to perform alternative functions.

(e) *RC5: complete loss of the reproductive organs*

RC5 represents the loss of both germline and somatic components of worker ovaries, i.e. ovarioles, oviducts, uterus and spermatheca. Although the developmental genetic basis of RC5 is largely unknown, we show that the full cluster of germ cells is eliminated during embryonic development in several species (figure 3*e*). This provides an indication of how the germline part of worker reproductive organs has been eliminated during development. RC4 and RC5 may be considered as different points along a continuum, but their underlying developmental processes are sufficiently different to warrant classifying them in different categories. First, RC4 involves a reduction of the germ cell number, whereas RC5 involves the complete removal of both germ cells and other somatic structures (whose development is independent from the germ cells). Second, RC4 leads to reducing the activity of the reproductive organs, whereas RC5 removes them altogether.

We hypothesize that RC5 is an adaptive trait that has evolved to eliminate worker reproduction and

reduce within-colony conflict. As discussed in RC4, there is a substantial genetic potential for completely eliminating reproductive organs. However, RC5 has only evolved in nine out of 283 ant genera. If RC5 were a consequence of neutral degeneration, we may expect the complete loss of worker reproductive organs to occur much more frequently in ant evolution. We have argued that alternative functions (trophic and/or physiological) are central for worker ovaries. For a species to evolve RC5, the cost associated with the ancestral reproductive function of worker ovaries should outweigh the benefit gained from maintaining worker ovaries for alternative functions.

5. REPRODUCTIVE CONSTRAINTS AND MULTI-LEVEL SELECTION

Our hypothesis that reproductive constraints represent adaptive mechanisms or traits that have evolved to actively maintain social harmony, rather than degenerative by-products of social selective forces, may be intuitively understood in a multi-level selection framework. Figure 5 attempts to summarize how the evolution of reproductive constraints is influenced by social selective forces and how they positively feed back to influence the social evolutionary dynamics of ant colonies. RC1–RC5 act to suppress worker reproduction at specific steps during development, thereby reducing within-colony competition. RC2–RC4 suppress or reduce the reproductive function of ovaries in a way that does not interfere with other alternative functions. RC2 (quantitative constraint) is a non-ultimate constraint that may result in either complete or partial blockage of the quantitative activity of worker ovaries depending on species. RC2 is a mechanism, whereas RC3 and RC4 are adaptive traits consisting of modifications of embryonic or larval development that results in the permanent loss or reduction of adult structures. RC1 (maternal determinant mis-localization) and RC5 (complete loss of reproductive organs) are both ultimate constraints that are fixed in the workers. RC5 suppresses both reproductive and alternative functions of the ovaries. RC1, however, is distinct from the other reproductive constraints because it represents a mechanism that blocks or reduces worker reproduction while restricting ovaries to performing alternative functions. Thus, reproductive constraints may reduce within-colony competition (reduced or no worker reproduction), increase cooperation between colony members (alternative functions) and therefore increase the ability of the colony to compete with other groups. Once they evolve, these constraints can positively feed back and may allow an increase in colony size and a more complex state of behavioural and morphological division of labour. Furthermore, they may render colonies robust to variations in their relatedness structure, potentially increasing their ability to cope with harsh environments (Korb & Heinze 2004; Smith *et al.* 2008; Wilson 2008*a*,*b*). A complete understanding of the roles of reproductive constraints in ant evolution will require integrating both ultimate (evolutionary) and proximate (developmental) causations. This socio-evo-devo approach holds much promise for gaining further insight into how cooperation, conflict and developmental systems evolve in social groups.

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