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Anarchy Is a Molecular Signature of Worker Sterility in the Honey Bee

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Abstract

Worker sterility is a defining characteristic of eusociality. The existence of the sterile worker caste remains a fundamental question for evolutionary biology as it requires the existence of genes that reduce personal reproduction. Currently, little is known about the proximate mechanisms underpinning worker sterility. Studies into a mutant "anarchistic" strain (in which workers can activate their ovaries) of honey bee, *Apis mellifera*, identified a list of candidate genes that regulate ovary activation. We quantified the expression of the four most promising candidate genes (*Anarchy*, *Pdk1*, *S6k*, and *Ulk3*) in nonactivated and activated ovaries of wild-type workers. Ovarian expression of *Anarchy*, a peroxisomal membrane protein, predicts the ovary state of workers with 88.2% accuracy. Increased expression of *Anarchy* in the ovary is strongly associated with suppression of oogenesis and its expression is sensitive to the presence of the queen. Therefore, *Anarchy* satisfies key criteria for a "gene underlying altruism". When we knocked down expression of *Anarchy* in the ovary using RNA interference (RNAi) we altered the expression of *Buffy*, a gene that regulates programmed cell death. Whole-mount multiplex fluorescent in situ hybridization (mFISH) shows *Anarchy* transcripts localize to degenerating oocytes within the ovary. Our results suggest that *Anarchy* is involved in the regulation of oogenesis through programmed cell death. The evolution of facultative worker sterility most likely occurred when the conserved mechanism of programmed cell death was co-opted to regulate ovary activation. *Anarchy* may therefore be the first example of a gene that has evolved through kin selection to regulate worker sterility.

Key words: **ovary activation, worker sterility, honey bee, anarchy, programmed cell death, oogenesis.**

Introduction

A defining characteristic of eusociality is an "altruistic" sterile or subfertile worker caste. The evolution of worker sterility implies the existence of genes that reduce personal reproduction. Based on theoretical modeling, Hamilton (1964a, 1964b, 1972) showed how alleles that impose a fitness cost on some individuals can spread in a population provided that they enhance the fitness of relatives that share the same allele. Half a century after Hamilton's seminal papers the molecular mechanisms underpinning worker sterility remain an enduring mystery.

An important first step to solving the mystery is to identify the genes that regulate worker fertility. These genes are predicted to have certain characteristics (Thompson et al. 2013). They should 1) increase the inclusive fitness of their carrier through kin selection (Hamilton 1964a, 1964b); 2) be differentially expressed between workers with nonactivated and activated ovaries; 3) show increased complexity in their downstream effects as sociality evolves; 4) only evolve socially mediated functions in groups of close relatives (Boomsma et al. 2011); 5) if caste-specific gene complexes develop, be co-expressed or genetically linked to caste-determining genes; 6) have, either currently or in the past, additive genetic effects and respond to selection; and 7) have multiple phenotypic effects that lessen the trade-offs involved in becoming sterile.

The honey bee (*Apis mellifera*) is a highly derived eusocial species and is the model organism for studies into eusociality. In adult workers, the reproductive state of the ovaries is plastic. The presence of the queen, secreting her queen mandibular pheromone, inhibits ovary activation in workers (Hoover et al. 2003). In the absence of a queen, workers are released from reproductive inhibition and activate their ovaries to lay male-destined eggs. Nevertheless, colonies are sometimes found in which the normally sterile workers activate their ovaries and lay eggs even when a queen is present (Oldroyd et al. 1994; Montague and Oldroyd 1998; Châline et al. 2002). Studies of these anarchistic workers have established that ovary activation has a strong genetic basis (Oldroyd et al. 1994; Montague and Oldroyd 1998; Châline et al. 2002; Thompson et al. 2006).

A mutant anarchistic strain of honey bee selected for a high level of worker reproduction (Oldroyd and Osborne 1999) has been an incisive tool for identifying some of the key genes that regulate the plasticity of the worker ovary between nonactivated and activated states. A mapping study of this strain identified a quantitative trait locus, *OvA3* located on chromosome one, that is significantly associated with ovary activation (Oxley et al. 2008). Additional support for this region was provided by an independent microarray study of wild-type workers comparing

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nonactivated and activated ovaries (Cardoen et al. 2011). Examination of this chromosomal region, in conjunction with gene functional annotation, allowed us to compile a short list of candidate genes (*Anarchy*, *Pdk1*, *S6k*, and *Ulk3*; supplementary table S1, Supplementary Material online) that putatively regulate the ovary state of the worker. *Anarchy* is the only gene that is located in the *OvA3* region and listed in the top differentially expressed genes between wild-type workers relative to anarchistic (Thompson et al. 2006). Based on sequence homology, Anarchy is a peroxisomal membrane protein from the mitochondrial solute carrier protein family (Visser et al. 2002). *Pdk1*, *S6k*, and *Ulk3* are all components of the highly conserved mechanistic target of rapamycin (mTOR) signaling pathway that controls cell growth and proliferation in response to environmental cues, such as nutrient availability (Chan and Tooze 2009; Laplante and Sabatini 2012). The mTOR pathway plays a role in female honey bee caste differentiation into a queen or a worker (Patel et al. 2007; Kamakura 2011; Mutti et al. 2011), so minor changes in the expression of the mTOR pathway genes may be sufficient to alter oogenesis. Thus, the mTOR pathway seemed likely to play a role in determining the ovary state in the worker.

Here, we investigate the effect of ovary state (nonactivated or activated) in wild-type adult honey bee workers on the expression of four candidate genes (*Anarchy*, *Pdk1*, *S6k*, and *Ulk3*) in the ovary. We experimentally manipulated the ovarian expression of *Anarchy*, the gene that best predicts ovary state, using RNA interference (RNAi) and thereby determine which cellular signaling pathways it influences to regulate ovary state. Then, we localized *Anarchy* in the ovary using whole-mount multiplex fluorescent in situ hybridization (whole-mount mFISH).

Results

The Candidate Gene *Anarchy* Is the Best Predictor of Worker Ovary State

We used Decision Tree Recursive Partitioning (Quinlan 1993) to determine which of the four candidate genes (*Anarchy*, *Pdk1*, *S6k*, and *Ulk3*) in the ovaries of 10- and 15-day-old wild-type workers, when oogenesis is underway (Koudjil and Doumandji 2008), optimally allocated samples to the nonactivated and activated ovary groups. The ovarian expression level of just the candidate gene *Anarchy* was sufficient to reach the optimal assignment and adding the ovarian expression level of the three other candidate genes into the analysis did not improve the assignment. Therefore, *Anarchy* showed the strongest association with worker ovary state. When the relative ovarian expression of *Anarchy* was greater than a threshold of 0.729, 16 of 16 samples were correctly assigned to the nonactivated ovary group. When the relative ovarian expression of *Anarchy* was less than or equal to a threshold of 0.729, 15 of 18 samples were correctly assigned to the activated ovary group. A cross-validation study showed that ovarian expression level of *Anarchy* alone correctly classified a worker's ovary state 88.2% of the time. The ovarian expression level of the three other candidate genes were poor predictors of worker ovary state: *Pdk1* 52.9%, *S6k* 55.9%, and *Ulk3* 55.9%.

Anarchy Is Likely to Be Associated with Programmed Cell Death

The strongest candidate gene for ovary state, *Anarchy* belongs to the mitochondrial solute carrier protein family, clustered within a clade of proteins that transport nucleotides (Palmieri 2013), whose members are involved in programmed cell death (Gutiérrez-Aguilar and Baines 2013). *Anarchy* is currently not well characterized so the mechanism by which it could regulate programmed cell death is unknown. We therefore investigated the expression of two additional genes (*Ark* and *Buffy*) that are known in *Drosophila melanogaster* (Peterson et al. 2007; Tanner et al. 2011) and the honey bee (Dallacqua and Bitondi 2014) to regulate programmed cell death during oogenesis. *Ark* is a pro-apoptotic factor that initiates the caspase cascade to induce cell death (Cain et al. 2002) and *Buffy* controls the mitochondrial pathway for programmed cell death (Tanner et al. 2011).

Principal component analysis (PCA) showed expression of the strongest candidate gene *Anarchy* in the ovaries of workers significantly covaries with expression of the programmed cell death gene *Buffy* ($r = 0.401$, $P = 0.002$, $n = 55$), the candidate gene *Pdk1* ($r = 0.354$, $P = 0.007$, $n = 56$), the candidate gene *Ulk3* ($r = 0.303$, $P = 0.025$, $n = 55$), and the programmed cell death gene *Ark* ($r = 0.279$, $P = 0.039$, $n = 55$) (supplementary fig. S1, Supplementary Material online). In combination, components 1 and 2 of the PCA explained 59.2% of the variance in gene expression.

When we expanded the Decision Tree Recursive Partitioning analysis to include the four candidate genes (*Anarchy*, *Pdk1*, *S6k*, and *Ulk3*) and the two programmed cell death genes (*Ark* and *Buffy*), the candidate gene *Anarchy* remained the gene most associated with worker ovary state, but the programmed cell death gene *Buffy* also had strong predictive power. The ovarian expression level of *Buffy* alone correctly classified a worker's ovary state 85.3% of the time.

Knockdown of *Anarchy* Alters the Expression of *Buffy*

To determine those cellular pathways that are influenced by the candidate gene *Anarchy*, we used RNAi to experimentally manipulate its expression in the ovaries of wild-type workers exposed to queen pheromone. At 12 and 24 h postinjection, there was a significant effect of treatment on the ovarian expression of *Anarchy* ($x^2_2 = 41.1$, $P < 0.001$ and $x^2_2 = 15.3$, $P < 0.001$, respectively) and expression was significantly reduced in the ovaries of workers treated with *Anarchy* double-stranded RNA (dsRNA) relative to the two controls (fig. 1*A* and *B*). The knockdown of *Anarchy* dissipated over time. In the ovaries of workers injected with *Anarchy* dsRNA, there was a significant increase in the expression of *Anarchy* between 12 and 24 h postinjection ($P = 0.016$). In contrast, in the ovaries of workers injected with the two controls there was no significant difference in the expression of *Anarchy* between 12 and 24 h

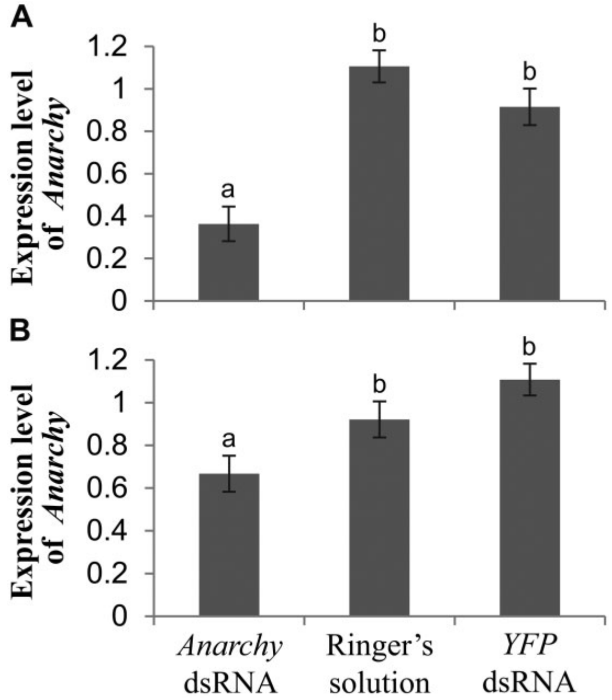

Fig. 1. Relative expression of *Anarchy* in the ovaries of workers (*A*) 12 and (*B*) 24 h postinjection of dsRNA solution for RNAi. Treatments: *Anarchy* dsRNA (target gene); Ringer's solution (procedural control); or *YFP* dsRNA (negative control). Sample size, see supplementary table S3*B*, Supplementary Material online. Error bars are standard error of the means. Different letters (a or b) represent statistically significant differences between samples, LSD post-hoc test ($P < 0.05$).

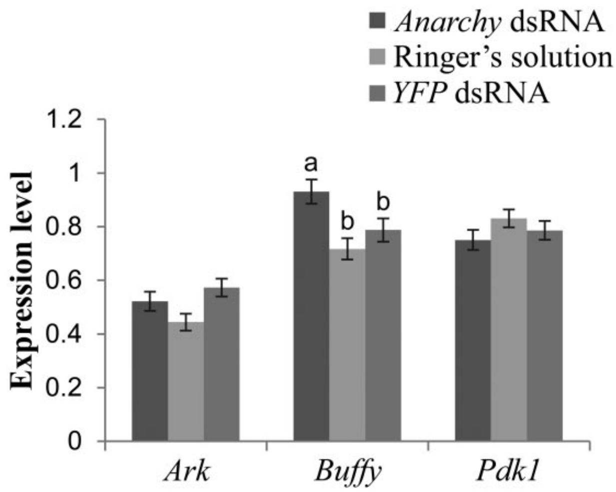

Fig. 2. Relative expression of the programmed cell death genes (*Ark* and *Buffy*) and mTOR pathway gene (*Pdk1*) in the ovaries of workers 24 h postinjection of dsRNA solution for RNAi. Treatments: *Anarchy* dsRNA (target gene) ($n = 9$); Ringer's solution (control) ($n = 11$); or *YFP* dsRNA (negative control) ($n = 9$). Error bars are standard error of the means. Different letters (a or b) represent statistically significant differences between samples, LSD post-hoc test ($P < 0.05$).

postinjection (Ringer's solution $P = 0.085$ and *Yellow Fluorescent Protein* [*YFP*] dsRNA $P = 0.31$).

We investigated whether knockdown of *Anarchy* in the ovary affected the expression of the two programmed cell death genes (*Ark* and *Buffy*) and the mTOR pathway gene that covaries the most with *Anarchy* (*Pdk1*), in the ovaries of 2-day-old workers. At 24 h postinjection, there was a significant effect of treatment on the ovarian expression of *Buffy* ($x^2_2 = 12.5$, $P = 0.002$; supplementary table S2*A*, Supplementary Material online). *Buffy* expression was significantly increased in the ovaries of workers treated with *Anarchy* dsRNA relative to the two controls (fig. 2). Any change in the expression of one programmed cell death gene can have profound effects on tissue homeostasis, as the balance between different apoptotic factors is important (Dallacqua and Bitondi 2014). A change in the expression of *Buffy* can either trigger apoptosis or inhibit apoptosis (Quinn et al. 2003; Wu et al. 2010). At 24 h postinjection, there was a significant effect of treatment on the expression of *Ark* ($x^2_2 = 8.0$, $P = 0.02$; supplementary table S2*A*, Supplementary Material online). However, this effect was driven by a treatment × replicate cage interaction and *Ark* is not significant if this interaction is removed from the model ($P = 0.29$).

Anarchy Localizes to Degenerating Oocytes

To determine where the candidate gene *Anarchy* and the programmed cell death gene *Buffy* transcripts localize in the wild-type worker's ovary, we used whole-mount mFISH. Exposure to queen pheromone causes workers to abort their developing oocytes, mid-oogenesis, resulting in the nonactivated ovary phenotype (Ronai et al. 2015). In nonactivated ovaries, *Anarchy* transcripts were located in degenerating oocytes and nurse cells (fig. 3*A*). In activated ovaries, *Anarchy* transcripts localized around the germinal vesicle (oocyte nucleus) of maturing oocytes (fig. 3*B*). At this stage of oogenesis, nurse cells undergo developmental programmed cell death (fig. 3*C*) after providing nutrients to the oocyte (Cavaliere et al. 1998). In both nonactivated and activated ovaries *Buffy* transcripts localized to the peritoneal epithelial sheath cells of the ovarioles (fig. 3*A* and *B*), the same location as is seen in the ovaries of worker larvae (Dallacqua and Bitondi 2014). In the ovaries of workers not exposed to queen pheromone, *Buffy* transcripts are also temporally localized to the pedicel, sometimes referred to as the basal stalk (supplementary fig. S2, Supplementary Material online), the same location as is seen in the ovaries of worker larvae (Dallacqua and Bitondi 2014). When *Buffy* is expressed in the pedicel of the developing ovaries of larvae, the ovariole is preserved from degeneration (Hartfelder and Steinbrück 1997). The expression of *Buffy* in the ovary of adult workers may therefore be a mechanism by which the oocytes are protected from degeneration. When we used *Anarchy* and *Buffy* sense riboprobes as mFISH negative controls, only background levels of these were detected in the ovaries (fig. 3*D*).

Anarchy Is Differentially Expressed between Worker Ovary States

We investigated whether there are any effects of reproductive state on the expression of the candidate (*Anarchy*, *Pdk1*, *S6k*,

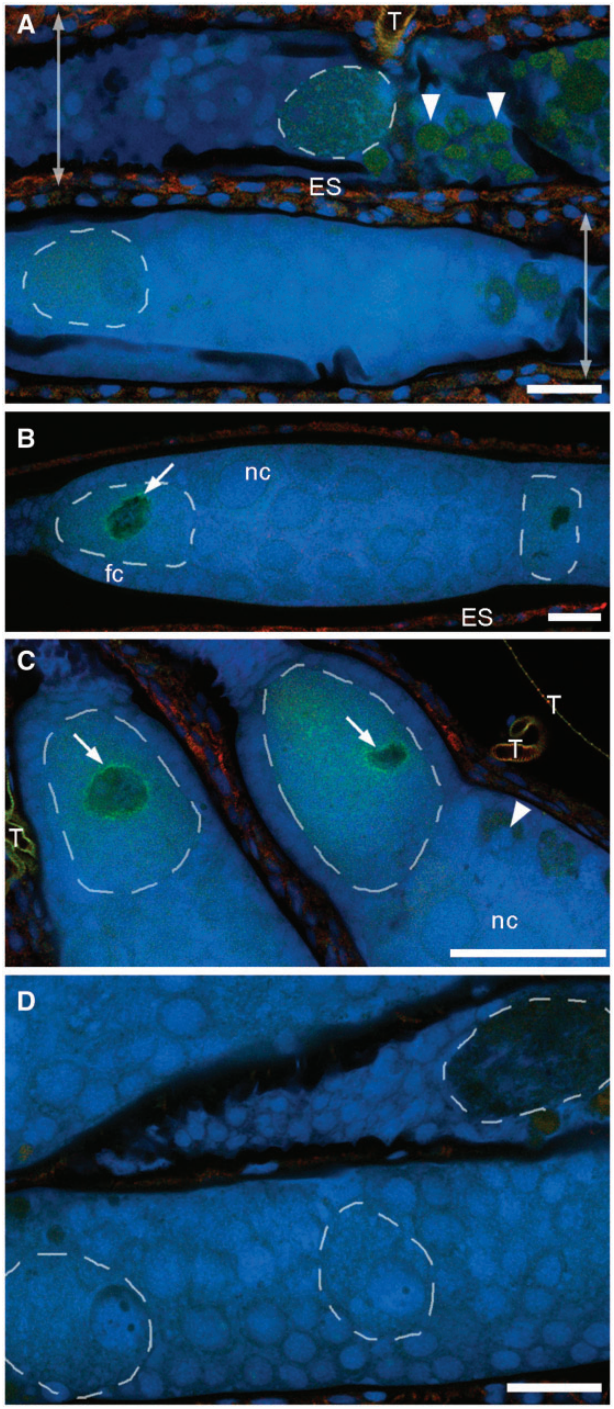

Fig. 3. *Anarchy* and *Buffy* transcript localization in ovaries of workers stained with the SYTOX Blue nucleic acid stain (blue). Oocytes are delineated by dashed lines and autofluorescent tracheoles (T) appear yellow. (*A*) Workers exposed to queen pheromone have nonactivated ovaries (ovarioles are delineated by double-headed arrows) which contain degenerating oocytes. *Anarchy* (green) localizes to the degenerating oocytes and nurse cells (arrowheads). The nurse cells have pycnotic nuclei, a marker of programmed cell death. *Buffy* (red) localizes to the peritoneal epithelial sheath cells (ES) of the ovarioles. (*B*) Workers not exposed to queen pheromone can have activated ovaries which contain developing follicles. Follicle cells (fc) surround the oocyte and its nutritive nurse cells (nc). *Anarchy* (green) localizes strongly at the germinal vesicle (arrow) of a maturing oocyte, this is not observed in the less mature oocyte to the right. *Buffy* (red) localizes to the peritoneal ES. (*C*) *Anarchy* (green) localizes strongly at the germinal vesicle (arrows) of maturing oocytes and the accompanying nurse cells are undergoing developmental programmed cell death (arrowhead). (*D*) An ovary labelled with *Anarchy* and *Buffy* sense riboprobes has only background levels of staining. Scale bars 20 μm.

and *Ulk3*) and programmed cell death (*Ark* and *Buffy*) genes in the ovaries of wild-type workers and queens. Workers of known age were collected from queenright and queenless colonies and their ovary state assessed. In queenless colonies, the proportion of workers with activated ovaries was 60% at 10 days and 75% at 15 days. Same-age sister queens were either virgins (all with nonactivated ovaries) or mated (all with activated ovaries).

Workers (10 and 15 days old) with nonactivated ovaries had significantly higher ovarian expression of *Anarchy*, *S6k*, and *Buffy* relative to workers with activated ovaries (supplementary table S2*B*, Supplementary Material online, and fig. 4*A*). Virgin queens had significantly higher ovarian expression of *S6k* and significantly lower ovarian expression of *Pdk1*, *Ark*, and *Buffy* relative to mated queens (supplementary table S2*C*, Supplementary Material online, and fig. 4*B*). Expression of *Anarchy* in the ovaries was not different between virgin and mated queens (supplementary table S2*C*, Supplementary Material online, and fig. 4*B*).

Queen Presence Only Affects the Expression of *Anarchy*

Of the three genes of interest that were associated with worker ovary state (*Anarchy*, *S6k*, and *Buffy*) only *Anarchy* had significantly different expression in the ovaries of queenright workers compared with queenless workers, irrespective of ovary state (supplementary table S2*D*, Supplementary Material online, and fig. 5*A*). Further, the expression of *Anarchy* in the ovaries decreased significantly in the ovaries of queenless workers as they aged (supplementary table S2*D*, Supplementary Material online, and fig. 5*A*)

Discussion

Thompson et al. (2013) argued that "genes underlying altruism", such as genes regulating worker fertility, should fulfil certain criteria. *Anarchy* satisfies all of the criteria that can be directly measured. First, the ovarian expression level of *Anarchy* predicts the ovary state of wild-type workers with an accuracy of 88.2%. Workers with nonactivated ovaries have more than 2-fold greater ovarian expression of *Anarchy* than workers with activated ovaries. An independent study has recently corroborated the relationship we have found between *Anarchy* and worker ovary state (Niu et al. 2014). *Anarchy* expression is also upregulated in the abdominal tissue of young wild-type workers relative to mutant anarchistic workers (Thompson et al. 2006) that have high rates of ovary activation. Therefore, increased expression of *Anarchy* in the ovaries is strongly associated with the termination of oogenesis. Second, the kin selected benefits of worker sterility only hold when a queen is present (Bourke 1988) as workers increase their inclusive fitness if they raise the offspring of the queen (their mother) than if they reproduce personally. Thus, a gene that regulates worker ovary activation must be

sensitive to the presence or absence of the queen in the colony. Expression of *Anarchy* in the ovary steadily decreases as queenless workers age (between 2 and 15 days old there is more than a 2-fold reduction in expression of *Anarchy*), whereas its expression in the ovary remains stable in queenright workers as degeneration of the oocytes is continuous. This suggests that *Anarchy* responds to the absence of the queen as soon as young workers emerge from the brood comb and is further evidence that *Anarchy* is associated with the termination of oogenesis. Expression of *Anarchy* in the fat body (Ament et al. 2011) and the brain (Grozinger et al. 2003) is also sensitive to queen presence. The mechanism by which the presence of the queen could affect gene expression in the ovaries of workers is unknown. However, components of queen mandibular pheromone are known to bind specifically to receptors that regulate signaling pathways in workers (Beggs and Mercer 2009). It is thus plausible that the social environment to which a worker belongs directly affects the transcription of its genes. Third, although a gene that regulates worker ovary activation must be responsive to queen pheromone in workers the same gene cannot be affected by queen pheromone in queens. (if it were queens would sterilize themselves with their own pheromone). The differing patterns of ovarian expression of *Anarchy* between worker and queen castes reflect this theoretical constraint. The fact that expression of *Anarchy* in the ovaries is stable in queens regardless of ovary state is further evidence that *Anarchy* is involved in the regulation of ovary activation in workers. Fourth, knockdown of *Vitellogenin* alters the expression of *Anarchy* in the fat body of adult workers (Ament et al. 2011). In honey bees, *Vitellogenin* is the paradigmatic example of a gene with multiple phenotypic effects, including being a major reproductive protein critical for oocyte maturation (Nelson et al. 2007). Vitellogenin is 11-fold lower in nonactivated ovaries compared with activated ovaries (Cardoen et al. 2012). We conclude that *Anarchy* fulfils the testable criteria for a gene regulating worker sterility (Thompson et al. 2013) and is the most promising gene involved in the regulation of worker sterility discovered thus far.

In contrast to *Anarchy*, the three other candidate genes (*Pdk1*, *S6k*, and *Ulk3*) do not satisfy key criteria for a gene regulating worker fertility (Thompson et al. 2013). Expression of the candidate genes that belong to the mTOR pathway are weak predictors of ovary state. Although expression of *S6k* is correlated with a worker's reproductive state, its expression is not sensitive to queen presence. In addition, expression of *S6k* is affected by reproductive state in both workers and queens, so its expression is not specific to the regulation of ovary activation in the worker caste. Our results therefore suggest that the mTOR pathway is unlikely to be directly involved in regulating worker ovary activation.

How might *Anarchy* regulate oogenesis in the worker? Programmed cell death is involved in regulating ovarian tissue across a diverse range of taxa (Hussein 2005; Tanner et al. 2011), including the honey bee (Tanaka and Hartfelder 2004; Tanaka et al. 2006; Cardoen et al. 2012; Cameron et al. 2013; Dallacqua and Bitondi 2014). The mouse ortholog of *Anarchy* is involved in apoptosis (Kannan et al. 2001) and

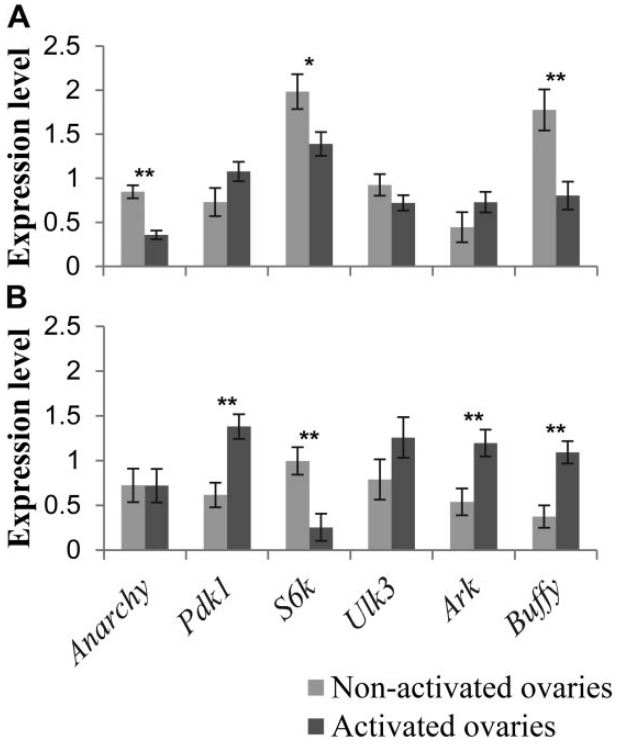

Fig. 4. Relative expression of the candidate genes (*Anarchy*, *Pdk1*, *S6k*, and *Ulk3*) and programmed cell death genes (*Ark* and *Buffy*) in the ovaries of (*A*) 10- and 15-day-old workers from queenless colonies with nonactivated ovaries ($n = 7$) and activated ovaries ($n = 15$); (*B*) 13-day-old virgin queens with nonactivated ovaries ($n = 6$) and mated queens with activated ovaries ($n = 6$). Error bars are standard error of the means. * indicates a significant difference between nonactivated and activated ovaries (*$P < 0.05$; **$P < 0.01$).

Anarchy belongs to a protein family that binds proteins from Buffy's family (Gutiérrez-Aguilar and Baines 2013). We suggest that *Anarchy* interacts with *Buffy*, which in turns regulates the mitochondrial cell death pathway (Tanner et al. 2011). In support of this view, we note that workers abort their developing oocytes in the presence of a queen (Ronai et al. 2015) and *Anarchy* transcripts are spatially restricted to these degenerating oocytes. *Anarchy* is likely to play a central role in regulating programmed cell death during oogenesis. We therefore propose that the programmed cell death pathway has been co-opted and modified through kin selection to become the central mechanism by which worker ovary activation is regulated in the honey bee.

Materials and Methods

Worker Study

Age-matched wild-type workers from two colonies of Australian commercial stock were obtained by incubating brood frames overnight. The following day, the workers were paint marked and then placed into one of two queenright (both queen and brood present) or two queenless (queen removed and brood present) nucleus colonies comprised four frames. At 2, 6, 10, and 15 days old, workers were collected from each of the colonies and immediately submerged in liquid nitrogen. At 10 days old, workers are

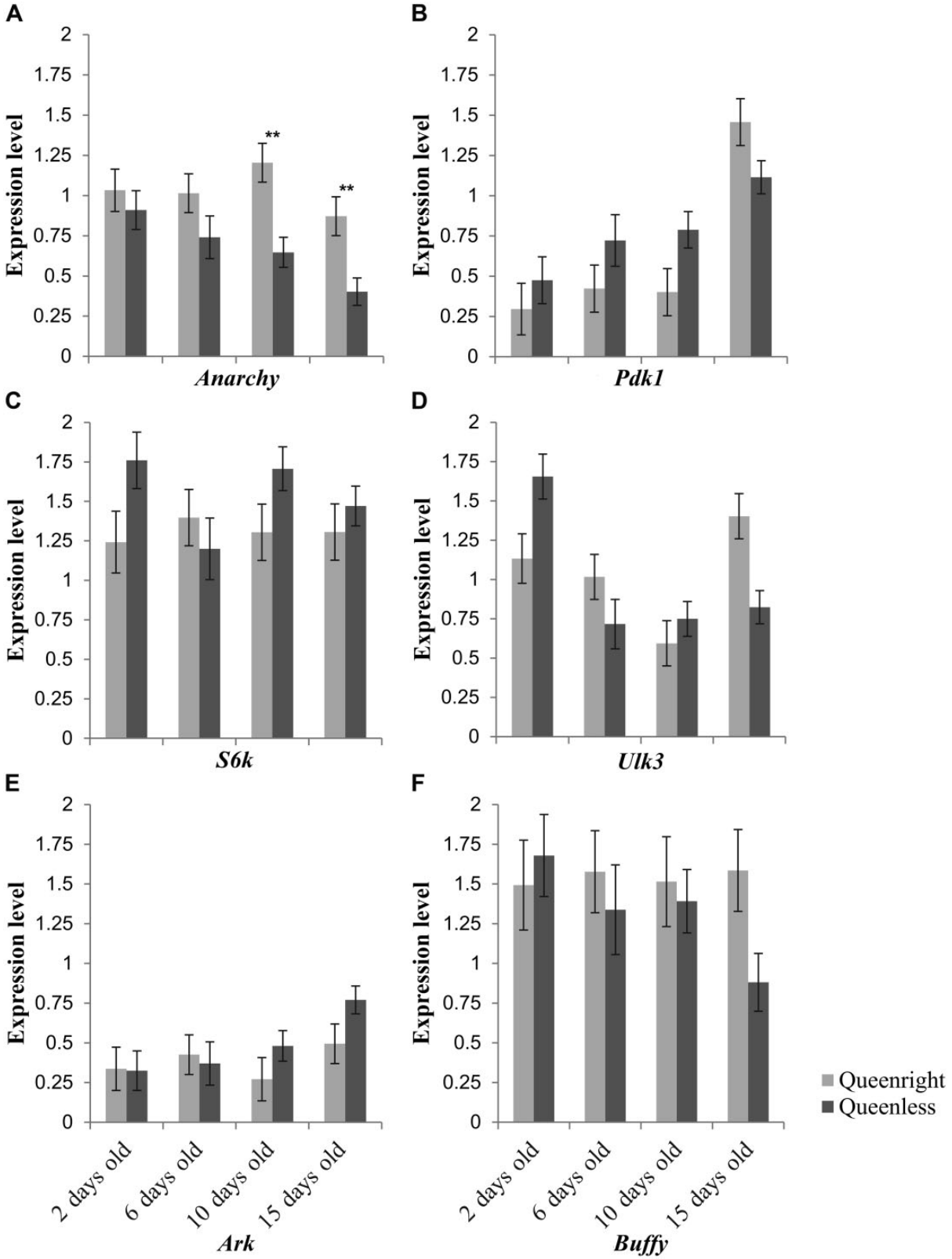

FIG. 5. Relative expression of the genes of interest in the ovaries of workers that were 2, 6, 10, or 15 days old from queenright and queenless colonies, irrespective of ovary state. (*A*) *Anarchy*, (*B*) *Pdk1*, (*C*) *S6k*, (*D*) *Ulk3*, (*E*) *Ark*, and (*F*) *Buffy*. Sample size, see supplementary table S3A, Supplementary Material online. Error bars are standard error of the means. ** indicates a significant difference between queenright and queenless according to LSD post-hoc tests ($P < 0.01$).

confined to the nest but by 15 days old some transition to outside tasks (Winston 1991).

Workers were dissected, their ovaries were removed, and then these were snap frozen. During dissection, the ovaries were scored for ovary state: nonactivated ovaries (transparent, thread-like ovaries) or activated ovaries (opaque, swollen ovaries) (Vergoz, Lim, and Oldroyd 2012). Each replicate sample consisted of five ovaries pooled according to queen state, age, and ovary state (sample sizes in supplementary table S3A, Supplementary Material online). Pooling was necessary to obtain a sufficient quantity of RNA.

Total RNA was extracted from ovary tissue samples using an RNAqueous-Micro Kit (Ambion). RNA concentration and purity were assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Ovary cDNA was synthesized using the Superscript RT-PCR (reverse transcription polymerase chain reaction) III kit (Invitrogen) with appropriate no RT controls. Primers were designed to quantify gene expression of the six genes of interest (*Anarchy*, *Pdk1*, *S6k*, *Ulk3*, *Ark*, and *Buffy*; supplementary table S1, Supplementary Material online). Following a previously established protocol (Vergoz, Lim, Duncan, et al. 2012) RT-quantitative real-time PCR reactions were performed using a LightCycler 480 (Roche Applied Science) with SYBR Green I Master Mix (Roche). The suitability of reference genes was assessed using the software *BestKeeper* (Pfaffl et al. 2004). *Actin* and *Rps5* were found to be suitably stable and both were used for normalization. The expression levels for the samples were calculated using the "relative quantification with efficiency correction method" (Pfaffl et al. 2004) and normalized to the geometric mean of the expression level of two reference genes providing a relative measure of gene expression (as described in Brito et al. 2010).

We used generalized linear models to examine the effects of queen state (queenright or queenless), age (2, 6, 10, or 15 days old), and ovary state (nonactivated or activated) on the expression of the genes of interest. Replicates are samples of five individual workers selected on the basis of the reproductive state of their ovaries. Where appropriate we compared individual treatments using Least Significant Difference (LSD) post-hoc tests.

Decision Tree Recursive Partitioning (Quinlan 1993) was used to identify which of the six genes' expression was most associated with worker ovary state (10 and 15 days old, regardless of queen presence). The model was constructed by first determining which gene's expression level optimally allocated samples to the nonactivated and activated groups based on a threshold of expression (algorithm J48 in Weka), that is, the threshold that maximized the correct assignment of the samples to the two groups. Then, depending on the accuracy of the first split, each group was split again into two subgroups using the expression level of the next most predictive gene, in an iterated process until the model reached an optimal assignment of samples to their correct group. The predictive power of these models was computed using 10-fold cross-validation to ensure an unbiased assignment. In the analyses presented here, one, or at most two genes were sufficient to reach the optimal assignment. Correlations between the six genes' expression levels across all samples (regardless of queen state, age, and ovary state) were examined using PCA and Pearson's correlations.

Queen Study

Sister virgin queens were reared and introduced as pupae into four-frame mating nucleus colonies. At 6 days old, the queens ($n = 12$) were randomly assigned to the virgin treatment (queen excluder strips were left in situ to prevent a mating flight) or the mated treatment (queen excluder strips were removed). At 13 days old, queens were collected.

Queens were dissected and their ovaries were removed. The ovaries differed in their reproductive state; virgin queens had nonactivated ovaries (underdeveloped ovaries, with no eggs) and mated queens had activated ovaries (large ovaries, with eggs) (Patrício and Cruz-Landim 2002). Each replicate sample consisted of the ovary from a single queen: Virgin queen ($n = 6$) and mated queen ($n = 6$). Gene expression of the six genes of interest was quantified as above. *Actin* and *Rps5* were found to be suitably stable reference genes and both were used for normalization. We used generalized linear models to examine the effect of ovary state (nonactivated or activated) on the expression of the genes of interest.

RNAi Worker Study

As is typical in genomic studies of social insects we attempted to minimize genetic and environmental heterogeneity among experimental units. Thus age-matched wild-type workers were obtained from a single colony. Workers ($n = 150$ per cage) were placed in six replicate laboratory cages. All cages were fitted with a small section of natural honey comb and a strip (0.5 queen equivalents) of queen mandibular pheromone (Phero Tech Inc., Canada) to simulate queen presence. The caged workers were provided with honey, ground pollen, and water ad libitum.

The gene that best predicted ovary state, *Anarchy*, was used for RNAi. We followed an established dsRNA protocol (Amdam et al. 2003). Primers were designed to amplify a 320-bp sequence of *Anarchy* (supplementary table S1, Supplementary Material online). *YFP* was chosen as a negative control dsRNA sequence because it has no homology to any gene of the honey bee. Primers were designed to amplify a 247-bp sequence of *YFP* (supplementary table S1, Supplementary Material online). The dsRNA was generated using the AmpliScribe T7-Flash Transcription Kit (Epicentre).

At 2 days old, workers were anaesthetized on ice and subsequently injected intra-abdominally with dsRNA solution (1 μl of 5 μg/μl) (Evans et al. 2013). For each cage, workers were randomly assigned to one of three treatments ($n = 50$ per treatment): *Anarchy* dsRNA as the target gene, *YFP* dsRNA as a negative control, and insect Ringer's solution as an injection procedural control. Workers of each treatment were paint marked with a different color.

At 12 and 24 h postinjection, workers were collected on dry ice from each cage ($n = 10$ per treatment) to determine whether expression of the target gene had been successfully

knocked down. Each replicate sample consisted of five ovaries pooled according to time postinjection, treatment group, and replicate cage (sample sizes in supplementary table S3*B*, Supplementary Material online). It is important to note that pooling dilutes any effects of knockdown, as we would not expect 100% efficiency of RNAi by injection.

Gene expression was quantified as above. *Ef1α* and *Rps5* were found to be suitably stable reference genes and both were used for normalization. First, we used generalized linear fixed effects models to examine the effect of time postinjection (12 and 24 h), treatment (*Anarchy* dsRNA, *YFP* dsRNA, and Ringer's solution), and replicate cage (1–6) on the expression of *Anarchy*. *Ef1α* was found to be a suitably stable reference gene and was used for normalization. Second, we used generalized linear models to examine the effect of treatment and replicate cage at 24 h postinjection on the expression of the genes *Anarchy* is most strongly correlated with (*Ark*, *Buffy*, and *Pdk1*). Where appropriate we compared individual treatments using LSD post-hoc tests.

Whole-Mount mFISH of Worker Ovaries

Age-matched wild-type workers were placed in four replicate laboratory cages (as described above) of which two were fitted with a queen mandibular pheromone strip to simulate queen presence. At 14 days old, workers were collected. During dissection, the ovaries were scored (as described above) for ovary state.

To localize the expression of *Anarchy* and *Buffy* in the adult worker ovary, we followed previously described methods (Zimmerman et al. 2013; Dallacqua and Bitondi 2014). *Anarchy* 320-bp antisense and sense riboprobes (supplementary table S1, Supplementary Material online) were conjugated to Alexa Fluor 488 and *Buffy* 372-bp anti-sense and sense riboprobes (supplementary table S1, Supplementary Material online) to Alexa Fluor 555 using the FISH Tag RNA Multicolor Kit (Invitrogen). Individual samples were four ovary pairs, and hybridizations were performed at least twice. Riboprobes were diluted in hybridization solution (queenright: *Anarchy* 0.4 ng/μl and *Buffy* 0.2 ng/μl; queenless: 0.4 ng/μl for both genes). Hybridization was carried out at 50 °C. Nuclei were stained with SYTOX Blue Nucleic Acid Stain (Invitrogen) (0.002%). Ovaries were examined under a Leica TCS SP5 II confocal laser scanning microscope (Leica microsystems). SYTOX Blue, Alexa488 and Alexa555 were excited by 458, 488 and 561 lasers, respectively, and emitted light was collected between 465 and 484, 500 and 550, and 580 and 650 nm. Images were collected sequentially.

Supplementary Material

Supplementary figures S1 and S2 and tables S1–S3 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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