e-ISSN 1734-9168
http://www.isez.pan.krakow.pl/en/folia-biologica.html

Folia Biologica (Kraków), vol. 73 (2025), No 1
https://doi.org/10.3409/fb_73-1.04

Is worker policing relaxed in honeybee families that experience a queenless period?

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Accepted April 16, 2025 *Published online April 29, 2025* *Issue online April 29, 2025*

Short communication

OSTAP-CHEC M., BAJOREK D., ŻMUDA A., STEC D. 2025. Is worker policing relaxed in honeybee families that experience a queenless period? Folia Biologica (Kraków) **73**: 30-35.

In honeybee colonies, the natural way of dividing an ever-expanding colony occurs through swarming. This phenomenon and the resulting temporary absence of the queen influence various colony parameters, such as changes in the development strategy of the larvae. In this study, we investigate whether families that have experienced a queenless period exhibit differences in their subsequent worker policing tendencies when compared to normal colonies. We analyse the removal rate of queen-laid and worker-laid male eggs in colonies that were temporarily orphaned and those with a constant queen presence. Our findings reveal inconsistent patterns across replications regarding the egg policing in colonies that experienced a queenless period. While the pooled data suggests that both types of eggs are policed slower in colonies that are temporarily orphaned, the analysis of individual replications reveals contradictory results – with this effect being strong in one replication, absent in the second and reversed in the third. Therefore, the rate of worker policing appears to be determined by colony-specific characteristics or other environmental factors, rather than by the temporary absence of the queen.

Key words: *Apis mellifera*, swarming, egg removal, orphaning.

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In the Western honeybee (*Apis mellifera* L.), swarming is the natural way to divide an ever-expanding colony. During swarming, the old queen leaves the native colony together with many workers, in search of a place for a new nest. The remaining bees care for the pupae, larvae and eggs of their younger worker cohorts and the developing new queens. However, before the new queen emerges, there is a period with no active queen present in the hive (Winston 1987). The absence of an adult-mated queen is quickly perceived by the workers, and this information is passed on to the larvae along with the food (Woyciechowski *et al.* 2017). This leads to a change in the development strategy of the larvae, with an increased investment in the reproductive organs (Woyciechowski & Kuszewska 2012).

Even in queenright conditions, some workers possess a reproductive potential and try to reproduce. At the same time, the workers impose a regime in which worker reproduction is suppressed. Worker policing is defined as any behaviour of the workers that restrains the reproduction of other workers (Ratnieks 1988). Most often, it means the selective removal of worker-laid eggs via oophagy

(Ratnieks & Visscher 1989; Ratnieks 1995) or aggression towards the workers attempting to lay eggs (Ratnieks & Visscher 1989; Visscher & Dukas 1995). This strategy is costly for the colony, as many male and female queen-laid eggs are removed by mistake (Kärcher & Ratnieks 2014). However, the effectiveness of worker policing is very high, because even though 7% of male eggs are worker-laid, only 0.1% of drones present within the colony are sons of the workers (Visscher 1989, 1996). This ensures the stability of the reproductive division of labour in the colony. One can imagine, however, that a queenless period after swarming leads to the development of workers with relaxed policing tendencies. This would be in line with their increased reproductive potential and the colony-level effects of the presence of such workers, including increased drone production (Kuszewska *et al.* 2018).

The aim of our study was to check whether families that have experienced a queenless period later differed in their worker policing tendencies, compared to families that did not go through such a period. We examined the rate of the removal of queen-laid and worker-laid male eggs in colonies that had previously been orphaned for a few days, and those in which the queen was always present. We used the rate of egg removal as a proxy for the intensity of worker policing, which is a commonly used method (e.g. Beekman & Oldroyd 2003; Pirk *et al.* 2002; Rojek *et al.* 2019). We hypothesised that families that experienced queenlessness would be characterised by relaxed policing when compared to normal families.

Material and Methods

The experiment was performed in three replicates (pairs of Colonies A and B), with a 3-4 day delay between the replicates. In all replicates, Colony A was the colony that experienced a queenless period, while B was the colony with the queen that was constantly present (normal). Colonies A and B were always similar in terms of their size, amount of supplies and health condition. In order to simulate a post-swarming condition, Colony A was divided into queenright and queenless subunits on Day 0. All the frames containing eggs or/and larvae were put into the queenless subunit. Colony B was not manipulated in any way. The subunits of Colony A were merged again on Day 12.

For the experiment, we used test frames that were comprised of both: (i) a fragment of a drone comb with worker-laid male eggs; and (ii) a fragment of a drone comb with queen-laid male eggs. One such frame was placed in Colony A and another in Colony B, above the queen excluder, on Day 38 (Figure 1). For all replicates of Colonies A and B, the worker-laid and queen-laid eggs were obtained from the same two unrelated colonies, following Pirk *et al.* (2002) and Beekman & Oldroyd (2003). On Day 38, the workers that were at their larval stage during the division of Colony A into subunits were at the age of 17-29 days. At that age, bees willingly undertake the removal of eggs (i.e. engage in policing, Ernst *et al.* 2017). We inspected the frames at time intervals (0, 1, 2, 4, 6, 24 h) to determine the egg survival rate. For this purpose, photographs of frames were taken using an Olympus TG3 camera at each time interval, and the numbers of eggs were counted from these photographs. The raw data from this counting is included in the Supplementary Materials (SM.01).

As mentioned above, the worker-laid and queen-laid eggs were sourced from other colonies. To obtain the fragments of combs with eggs for testing, we used frames which consisted of four removable

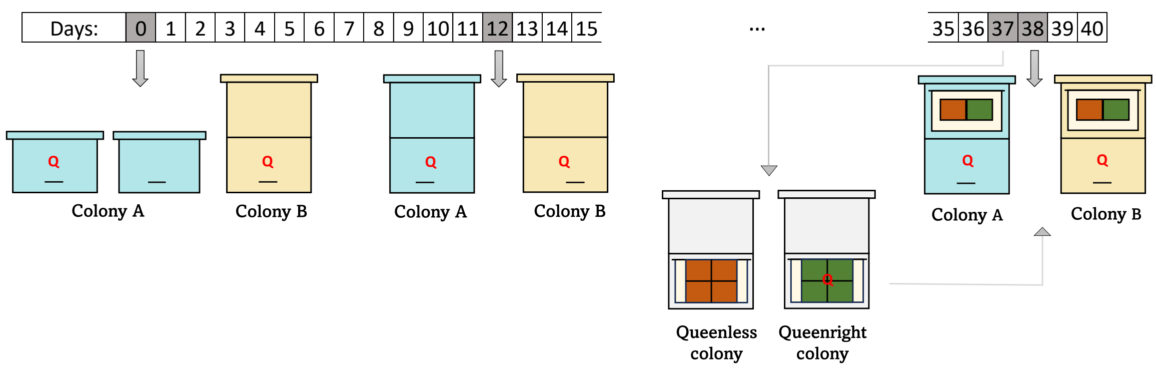

Fig. 1. Schematic design of the experimental manipulation in the apiary depicting the whole period for a single replication. Day 0 – division of Colony A into queenless and queenright subunits; Day 12 – merging subunits of Colony A; Day 37 – experimental frames placement in the queenless and queenright colonies, respectively; Day 38 – testing frames placement (with the fragments of worker-laid and queen-laid male eggs) in Colonies A and B, respectively.

fragments of drone combs each. These frames were placed in the colonies designated for egg production on Day 37 of each replication. One frame was placed in a colony that was queenless for 37 days (for the worker-laid eggs) and another frame was in a queenright colony, inside an insulator with a queen (for the queen-laid eggs). On Day 38, the frames with drone eggs were extracted from the hives and two fragments per each frame, which comprised the highest number of queen-laid and worker-laid eggs, were taken for testing. The frames used for testing in Colonies A and B were assembled in a way so that each was comprised of one fragment with queen-laid and one fragment with worker laid-eggs.

Statistical analyses

To analyse the general patterns present in the egg removal rate, we used a mixed-effects Cox proportional hazard regression ('coxme' function in the 'survival' package in R; R Core Team 2021). This model included two fixed factors: colony type (A vs B) and egg type (worker-laid vs queen-laid), as well as their interactions, and a random factor 'replicate'. In the next step, we performed separate analyses for the worker-laid and queen-laid eggs using the same method, comparing their removal rates depending on the colony type (fixed factor: Colony A vs Colony B), with the random factor 'replicate' included. However, because we observed a large variance in the worker-laid egg removal across the replicates, we also conducted separate Cox regressions ('coxph' function in the 'survival' package in R; R Core Team 2021) for each replicate and each egg type. In these analyses, only the fixed factor of the colony type (Colony A vs Colony B) was included. To illustrate the survival patterns in each colony and for each egg type, six Kaplan-Meier survival curves were generated using the 'survfit' function from the 'survival' package in R (R Core Team 2021), based on the raw egg survival data. The R script with the analysis code (SM.02) and the dataset used in the analyses (SM.03) are provided as Supplementary Materials.

Results

The overall egg removal rate was lower in families where the workers had experienced a queenless period than in normal families ($z = -6.74$, $p < 0.001$). The worker-laid eggs had a 6.75 times higher risk of removal than the queen-laid eggs ($z = 29.72$, $p < 0.001$). The interaction between the colony type and egg type was significant, indicating that the effect of queenlessness on egg removal differed between the egg types ($z = 4.98$, $p < 0.001$). Specifically, the difference in removal rates between the queen-laid and worker-laid eggs was smaller in colonies that had experienced queenlessness than in normal colonies.

When analysing the removal rates separately for the queen-laid and worker-laid eggs, both showed a lower removal rate in families with workers that had experienced a queenless period compared to normal families (queen-laid: $z = -7.99$, $p < 0.001$; worker-laid: $z = -2.31$, $p = 0.021$). About 60% of queen-laid eggs survived in the queenless colonies (A) compared to 40% in the normal colonies (B). For the worker-laid eggs, almost all were removed on the same day that they appeared, with only 29 surviving 24 hours (~1% of all placed). Additionally, the inclusion of replicate as a random effect in both models significantly explained the variation in egg removal rates (worker-laid: $\chi^2 = 187.26$, $p < 0.001$; queen-laid: $\chi^2 = 138.28$, $p < 0.001$), suggesting that the replicate's identity contributed to differences in the egg removal patterns.

When analysed replicate-by-replicate, we observed varying patterns. In the first replicate, both queen-laid and worker-laid eggs were removed at a higher rate in the family with workers that experienced a queenless period compared to the normal family (queen-laid: $z = 4.60$, $p < 0.001$, HR = 2.034, 95% CI [1.503, 2.754]; worker-laid: $z = 3.90$, $p < 0.001$, HR = 1.413, 95% CI [1.188, 1.681]). However, this pattern was reversed in the third replicate (queen-laid: $z = -10.47$, $p < 0.001$, HR = 0.181, 95% CI [0.132, 0.250]; worker-laid: $z = -9.37$, $p < 0.001$, HR = 0.510, 95% CI [0.443, 0.587]); while in the second replicate, there was no significant difference (queen-laid: $z = -1.44$, $p = 0.151$, HR = 0.744, 95% [0.496, 1.115]; worker-laid: $z = 0.48$, $p = 0.632$, HR = 1.035, 95% CI [0.8998, 1.190]). Despite a general trend suggesting relaxed policing in queenless families, a closer analysis revealed differing patterns across the replicates, with increased (replicate I), decreased (replicate III) or no differences (replicate II) in the egg removal rate compared to normal families (Figure 2). The minimal overlap in the confidence intervals across the replicates highlights the fact that each repetition exhibited a completely different pattern, suggesting that other factors or colony-specific characteristics play a much greater role in this effect than past queenlessness.

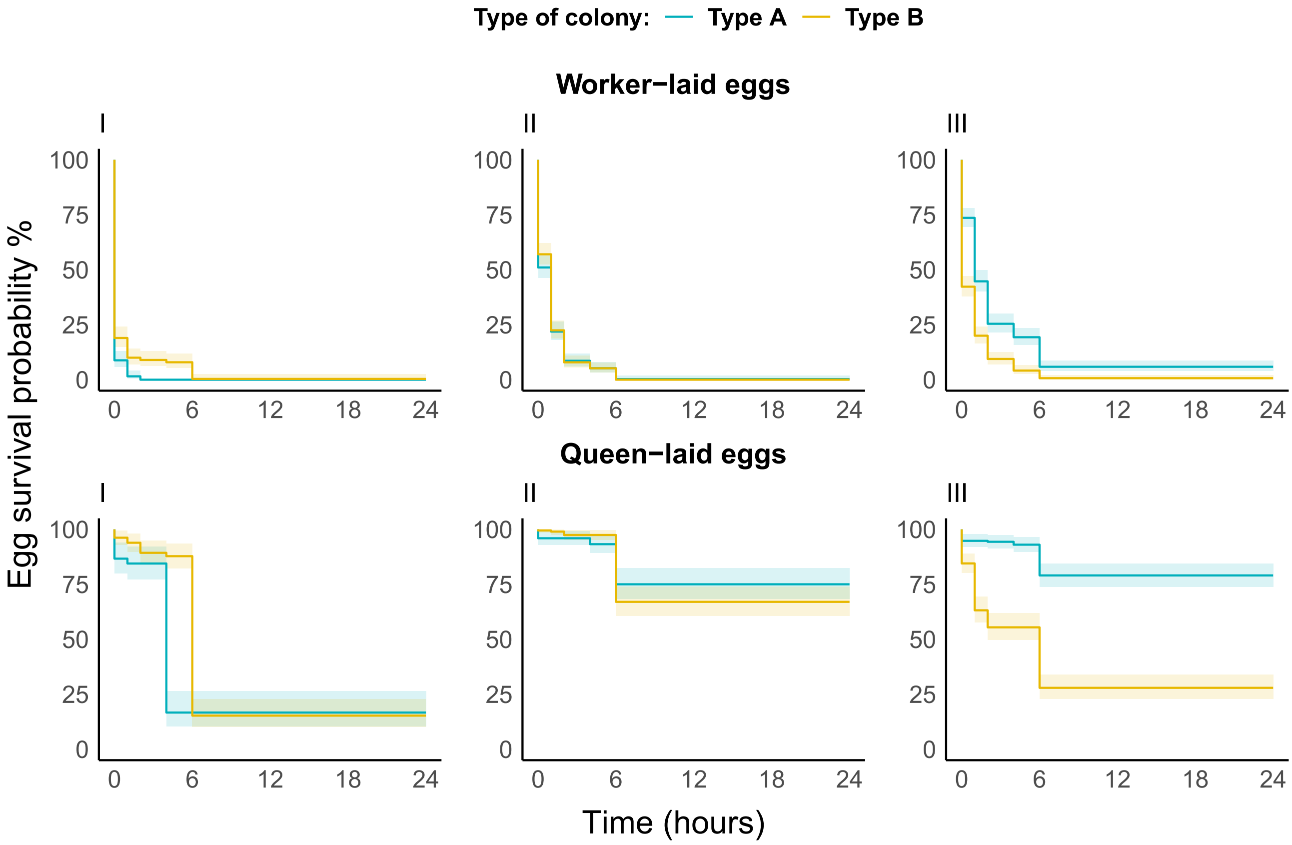

Fig. 2. Worker-laid (first row) and queen-laid (second row) male egg survival rates presented as Kaplan-Meier survival curves, with the shaded areas representing model-derived 95% confidence intervals. Results are shown for colonies that experienced a queenless period in the past (A) and colonies in which the queen was permanently present (B) across replications I, II and III.

Discussion

Our results confirm previous observations that worker policing is extremely effective (Visscher 1989, 1996) and persists even in colonies that have experienced a period of functioning without a queen. In all the colonies, almost all worker-laid eggs were removed after 6 hours, while the worker policing against queen-laid male eggs was lower but still considerable in particular colonies. Between 15% and 80% of the queen-laid male eggs remained after 24 hours, depending on the colony. These results show that policing workers make frequent mistakes and remove queen-laid eggs much more often than has been previously suggested (Kärcher & Ratnieks 2014). Both the results obtained in our study – namely, those regarding no apparent effect of a history of queenlessness on worker policing, and a surprisingly high rate of the removal of queen-laid eggs – mimic those of Rojek and their co-authors (Rojek *et al.* 2019). Moreover, in their study, the authors compared the removal rate of eggs laid by workers characterised by a normal or increased reproductive potential, as well as eggs laid by queens from the same or different nests. The results did not reveal any differences in the rate of egg removal, either between the two types of worker-laid eggs or between the two types of queen-laid eggs. However, it was observed that in certain colonies, the overall rate of egg removal was significantly higher than in others (Rojek *et al.* 2019). Our results, along with those of Rojek and co-authors, suggest that the level of worker policing is a specific characteristic of a given colony.

In colonies experiencing a queenless period, the workers can change some developmental strategies and behaviours, e.g. by investing more in reproductive organs or engaging less in the care of offspring (Woyciechowski & Kuszewska 2012). However, queenlessness seems not to impact the activity of worker policing, as it showed different patterns in each replicate. One possible explanation for the variation in the worker policing rates observed between colonies is that this behaviour is a specific feature of a given colony. From the perspective of the kin selection theory, such colony-specific patterns could be driven by the genetic relatedness among workers. The degree of kinship between nestmates is known to influence cooperative behaviours such as social grooming or trophallaxis (Frumhoff & Schneider 1987), and it is also likely to shape the intensity of worker policing. In colonies where the queen has mated multiple times, the average relatedness among workers is lower; thus, the kin selection theory predicts a higher rate of worker policing (Woyciechowski & Łomnicki 1987). Such a heightened policing may also increase the likelihood of mistakenly removing queen-laid eggs (Kärcher & Ratnieks 2014). In contrast, in colonies where the queen has mated only a few times and the worker relatedness is higher, policing is expected to be less intense (Woyciechowski & Łomnicki 1987). This theory finds support in a comparative analysis of the worker policing and male parentage across 109 species of ants, bees and wasps, confirming a higher occurrence of worker policing in species where the workers exhibit greater relatedness to the queen's sons compared to the sons of other workers (Wenseleers & Ratnieks 2006).

Nevertheless, although this pattern has evolved across different species, it seems unlikely that individual workers are able to assess and adjust their policing behaviour in response to real-time changes in the colony relatedness. Loope *et al*. (2013) tested whether workers in single-patriline colonies tolerated worker-derived males or relaxed their policing, but found no such effect. Instead, worker-laid eggs were policed just as strictly in colonies with singly-mated queens as in those with multiply-mated queens. This suggests that the policing intensity may not flexibly respond to the kin structure. The variations observed in our replicates may still reflect colony-specific characteristics, but this likely relates more to factors such as the colony strength or resource availability. Additionally, as each replicate included only a single measurement, we cannot exclude the influence of random factors such as the weather conditions, stochastic egg mortality or other environmental variables specific to the day of the measurement.

Plausibly, our results indicate a different direction to the effect of past queenlessness on the rate of worker policing because they reflect other family characteristics that were likely not accounted for. Given its scale and the far-reaching consequences of swarming, finding no uniform pattern was surprising. Among the consequences of past queenlessness are increased drone production (Kuszewska *et al.* 2018) and heightened worker ovariole activeness (Holmes *et al.* 2014). It also leads to worker drift to foreign colonies, which allows them to reproduce in those colonies rather than in their own (Kuszewska *et al.* 2018). Furthermore, swarming has a direct impact on the age structure of the colony. As a significant proportion of young bees depart with the swarm, many older bees must adhere to their brood care responsibilities, resulting in physiological changes (Wegener *et al.* 2009). Considering all the documented changes that occur in a colony during the queenlessness resulting from swarming, the absence of an effect on the rate of egg removal appears remarkable. Although our experimental setup did not fully replicate natural swarming conditions – since the same queen returned after the queenless period – we created queenless conditions similar to those associated with swarming, where the queen's sudden absence and the departure of some workers leads to a cessation of egg laying. This setup has been commonly used in past research focused on the consequences of swarming (Woyciechowski & Kuszewska 2012; Rojek *et al*. 2019).

In summary, our study demonstrates that periods of queenlessness do not affect the level of worker policing in honeybee colonies, both with regard to worker-laid and queen-laid eggs. Although the overall result may suggest such a relationship, the analysis of individual replicates reveals no consistent effect. The obtained result is surprising, considering the numerous documented effects associated with swarming and the temporary absence of a queen. However, our results indicate that the rate of egg removal is more likely driven by colony-specific factors or environmental conditions present on a given day, rather than being a direct consequence of temporary queenlessness.

Acknowledgements

We are grateful to Krzysztof Miler (ISEA PAS) for a critical reading and suggestions regarding our manuscript.

Author Contributions

Research concept and design M.O-Ch., D.S.: Collection and/or assembly of data: M.O-Ch., D.B., A.Ż., D.S.; Data analysis and interpretation: M.O-Ch., D.B.; Writing the article: M.O-Ch.; Critical revision of the article: M.O-Ch., D.B., A.Ż., D.S.; Final approval of article: M.O-Ch., D.B., A.Ż., D.S.

Conflict of Interest

The authors declare no competing interests.

Supplementary Materials

Supplementary Materials to this article can be found online at:
http://www.isez.pan.krakow.pl/en/folia-biologica.html
Supplementary files:

SM.01 – Raw survival data for worker-laid and queen-laid eggs.

SM.02 – R script with the code used for the survival analyses.

SM.03 – Dataset used in the statistical analyses.

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