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Differences in the suitability of published honey bee (*Apis mellifera*) reference genes between the African subspecies *Apis mellifera scutellata* and European derived *Apis mellifera*

Anja Buttstedt | **Christian W. W. Pirk** | **Abdullahi A. Yusuf**

Social Insects Research Group, Department of Zoology and Entomology, University of Pretoria, Pretoria, South Africa

Correspondence
Anja Buttstedt, Social Insects Research Group, Department of Zoology and Entomology, University of Pretoria, Private Bag X20, 0028 Hatfield, Pretoria, South Africa.
Email: anja.buttstedt@gmail.com

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Abstract

Quantitative real-time polymerase chain reaction (qPCR) is a method widely used to determine changes and differences in gene expression. As target gene expression is most often quantified relative to the expression of reference genes, the validation of suitable reference genes is of critical importance. In practice, however, such validation might not be thoroughly conducted if the same species and the same tissue or body parts are used for qPCR experiments. Here we show, that qPCR reference genes published for workers of European honey bee (*Apis mellifera*) subspecies fail to be stably expressed in workers of the African subspecies *Apis mellifera scutellata*. This is the case even when the sampled workers are in the same life stage, the same organ was dissected and the same reagents were used. Thus, reference genes need to be thoroughly re-tested before they can be used as suitable references even when the only thing that changes is the subspecies used.

KEYWORDS
A. mellifera subspecies, quantitative real-time PCR, reference genes, *scutellata*

1 | INTRODUCTION

The finding that messenger-RNA can be amplified after reverse transcription by polymerase chain reaction (PCR) opened new possibilities to quantify transcripts (Newman et al., 1988; Rappolee et al., 1988). Combined with the discovery that the increase in PCR product can be monitored with the help of DNA binding dyes, whose fluorescence increase upon binding double-stranded DNA (Higuchi et al., 1992, 1993), this enabled the development of quantitative real-time PCR (qPCR) (Wittwer et al., 1997). Quantitative real-time PCR is since then widely used to quantify transcripts and detect differences and changes in gene expression (Bustin et al., 2009; Pfaffl, 2001). Target gene expression is most often quantified relative to the expression of one or more reference genes which should not be regulated under the experimental conditions and be constantly expressed over all considered samples (Bustin et al., 2009; Pfaffl, 2001). Genes that are often used as reference genes are so called housekeeping genes, which fulfil fundamental cellular functions and are expressed in virtually all cell types (Alberts et al., 2002). The expression of housekeeping genes is incomprehensibly often considered to be relatively stable compared to other genes, although there is overwhelming evidence for decades that they are very often differentially expressed between tissues, developmental/life stages, treatments and timepoints within a treatment (Butte et al., 2001; Dheda et al., 2004; Goldsworthy et al., 1993; Thellin et al., 1999). Thus, genes that have been published as reference genes should only be considered as stable for the particular experimental situation, and validation is of critical importance for each individual experiment (Bustin et al., 2009; Dheda et al., 2004; Pfaffl, 2001). There are currently different software packages available to test the suitability of a gene as a

reference gene. Such software pages include the likes of GeNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004) and RefFinder (Xie et al., 2023), all of which are ultimately based on the extent to which the measured quantification cycle (C_q) values vary between samples. In general, any studied reference gene with a quantification cycle (C_q) standard deviation higher than 1 has to be considered as inconsistent and excluded as reference gene (Pfaffl et al., 2004).

The Western honey bee *Apis mellifera* is one of the best studied insect species and thus a variety of publications exist validating reference genes for qPCR (Jeon et al., 2020 [*A. mellifera ligustica* hybrids, Republic of Korea]; Kohútová et al., 2013 [*A. mellifera carnica*, Slovakia]; Lourenço et al., 2008 [Africanized *A. mellifera*, Brazil]; Moon et al., 2018 [*A. mellifera ligustica* hybrids, Republic of Korea]; Scharlaken et al., 2008 [*A. mellifera carnica*, Belgium]; Wieczorek et al., 2020 [*A. mellifera carnica*, Poland]). Our research concentrates, among other things, on hypopharyngeal glands (HPGs), located in the heads of honey bee workers, which synthesise proteins to be secreted into larval food jelly (Patel et al., 1960). As the HPGs are primarily active in young workers that feed the larvae, finding reference genes is especially challenging when comparing gene expression, for example in honey bee heads among queens, drones and workers or in HPGs of workers of different life stages and we always validated reference genes thoroughly for new qPCR studies (Buttstedt et al., 2013; Dobritzsch et al., 2019; Winkler et al., 2018).

Apis mellifera is divided into several subspecies distributed across the native range in Europe, Africa and western Asia, based on their morphology, genetics, behaviour, ecology and physiology (Han et al., 2012; Hepburn & Radloff, 1998; Mumoki & Crewe, 2021; Ruttner, 1988). We here report on the non-suitability of reference genes published for workers of European *A. mellifera* sampled in Halle (Saale), Germany (mix of at least three subspecies) (Dobritzsch et al., 2019) for studies using workers of the African subspecies *A. mellifera scutellata* sampled in Pretoria, South Africa. This is particularly interesting, as all variables, like tissue, bee life stage, reagents, qPCR thermocycler as well as experimenter, were kept the same. Therefore, published reference genes should not only be revalidated when the experimental conditions change but also when the same experiment is performed in another subspecies or population.

2 | MATERIALS AND METHODS

Age matched *A. mellifera scutellata* workers were sampled in February and March 2021 from a colony headed by a naturally mated queen located in Pretoria, South Africa after 0 (directly after hatching), six (brood raising nurse bees with developed hypopharyngeal glands [HPGs]) and 26 days (foraging bees with undeveloped HPGs). Dissections of the HPGs were performed in RNA later, RNA was extracted according to the manufacturer's protocol (NucleoSpin® RNA Kit, Macherey-Nagel, Düren, Germany) and RNA concentration was photometrically determined using a Nanodrop 1000 (Thermo Fisher Scientific). 200 ng RNA were reversed transcribed in a total of 15 µL using 0.2 µg oligo(dT)$_{15}$ primer, 1.2 µL dNTPs (10 mM each) and 100 units M-MLV reverse transcriptase (all Promega). cDNA was purified using the NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel). The concentration was set to 10 ng/µL and cDNA from three individuals was pooled. Five pools per age (day 0, 6 or 26) were analysed resulting in a total of 15 samples for subsequent qPCRs, which were performed as described in Dobritzsch et al. (2019) (SensiMixTM SYBR® No-ROX Kit, Meridian Bioscience) in a CFX96™ real-time PCR thermocycler (Bio-Rad). Quantitative cycle (C_q) values were determined with the Bio-Rad CFX Manager 2.1 using linear regression for each sample. Gene-specific primers were ordered from Inqaba Biotec (Pretoria, South Africa). C_q values for age-matched (day 0, 8 and 24) *Apis mellifera* worker HPGs sampled in Halle (Saale), Germany were adopted from Dobritzsch et al. (2019). Reagents (NucleoSpin® RNA Kit, NucleoSpin® Gel and PCR clean-up kit, oligo(dT)15 primer, dNTPs, M-MLV reverse transcriptase, SensiMixTM SYBR® No-ROX) were ordered from the same companies, the qPCR thermocycler was the same model and the experimenter (AB) was the same in both studies. However, differences between the studies, in addition to honey bee subspecies besides general climate (Cwa; warm temperate, winter dry, hot summer in this study; Cfb; warm temperate, fully humid, warm summer in Dobritzsch et al., 2019 (climate classification based on Kottek et al., 2006) and floristic region (sudano-zambezian in this study, circumboreal in Dobritzsch et al., 2019 (floristic regions based on Takhtajan, 1986), still occurred and were as follows: primer manufacturers, water used for the reactions, seasons (late summer/early autumn in this study, late spring/early summer in Dobritzsch et al., 2019) and specific age for nurse and forager honey bee life stage (day 6 and 26 in this study, day 8 and 24 in Dobritzsch et al., 2019). The 2 days earlier sampling for South African nurse bees was decided upon as it has been shown that HPG development in *A. mellifera scutellata* peaks at day 6 which is earlier than in European honey bees (Crailsheim & Stolberg, 1989 [*A. mellifera carnica*, Austria]; Langlands et al., 2022 [*A. mellifera scutellata*, South Africa, Pretoria]). The 2 days later sampling of *A. mellifera scutellata* forager bees was decided based on weather.

Statistical analyses were performed with STATISTICA 14.0 (StatSoft). C_q values were normally distributed (Shapiro Wilk test, $p = .81$) and analysed via one-way analysis of variance (ANOVA) with post hoc Bonferroni tests and via a general linear model (GLM). RNA amounts were not normally distributed (Shapiro Wilk test, $p < .0001$) and analysed via Kruskal–Wallis ANOVA with post-hoc pairwise comparisons.

3 | RESULTS AND DISCUSSION

The two reference genes used in Dobritzsch et al. (2019), *proteasome subunit beta type-1* (*pros26*) (primers: F-GCTGATAATGGAGG GAGTGTCA, R-CCAACAACCTGAGCAACCCA) and *major royal jelly protein 8* (*mrjp8*) (primers: F-TGGACTCAAGCATCGGCTAA, R-TGGCAACCACTTCGATATTTCTT) showed standard deviations

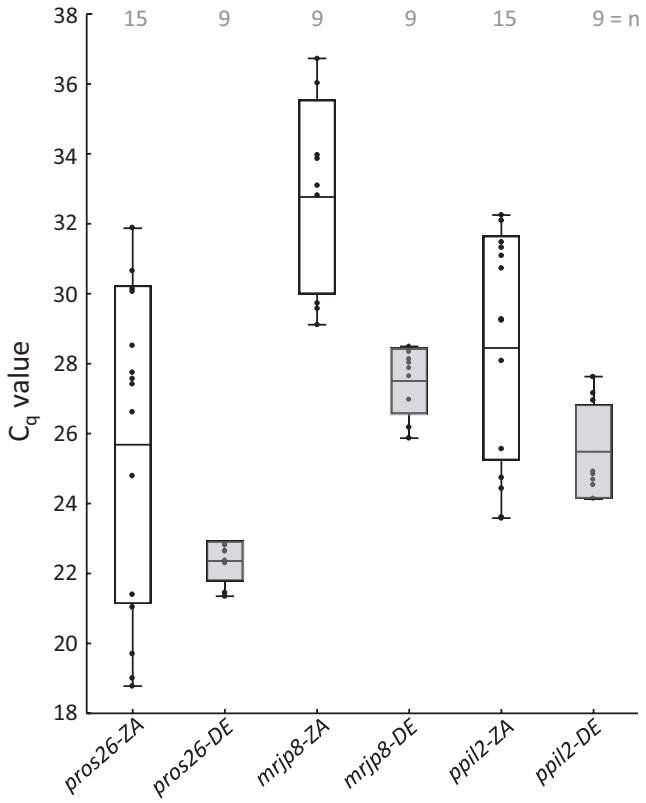

FIGURE 1 C_q values of validated reference genes. Boxes show mean ± standard deviation and whiskers show min-max. Individual data points are shown as circles. White boxes, ZA = *A. mellifera scutellata* sampled in South Africa; grey boxes, DE = *A. mellifera* sampled in Germany. Sample size (*n*) refers to pools of HPGs of 3 workers each.

below 1 across all HPG samples of European *A. mellifera* (DE-*pros26*, SD = 0.58; DE-*mrjp8*, SD = 0.95). However, in HPGs of South African *A. mellifera scutellata* SDs were for both genes much higher (ZA-*pros26*, SD = 4.56; ZA-*mrjp8*, SD = 2.78) (Figure 1). This is a 7.9- and 2.9-times SD difference for *pros26* and *mrjp8*, respectively.

That some published reference genes are not suitable for HPGs during a worker honeybees' life from hatching until the late forager period has already been noticed earlier by us (Dobritzsch et al., 2019). Genes that had been proven stable before in workers' heads, like the *ribosomal protein S5α* (*rpS5α*) (Buttstedt et al., 2013) and the *peptidyl-prolyl cis-trans isomerase-like 2* (*ppil2*) (Winkler et al., 2018) had to be excluded when dissected HPGs were considered (Dobritzsch et al., 2019). However, we tested *ppil2* (primers: F-TTTAATGCGGCACACTATTCTACT, R-AACTGCTGCTTGATGTG TAGTTTC) in *A. mellifera scutellata* with the same result: SD in *A. mellifera scutellata* was 2.4-times higher than in the European *A. mellifera* (ZA-*ppil2*, SD = 3.21; DE-*ppil2*, SD = 1.35). Besides these three genes for which we had direct comparative values in HPGs of European *A. mellifera*, we tested five further published reference genes which have been recommended for usage in heads across honey bee life stages (nurse or forager) and seasons (*A. mellifera ligustica* hybrids, Republic of Korea) or which have been shown to be stably expressed in *A. mellifera scutellata* heads (*cytochrome P450 4g11 (cyp4g11)* (primers: F-GGCTGTAATGAAGATGTGCGAC, R-GTGCG CTATTATCAATGATGTTACG); *eukaryotic translation initiation factor 3 subunit C* (*eIF3-S8*) (primers: F-TGAGTGTCTGCTATGGATTGCAA, R-TCGCGGCTCGTGGTAAA); *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) (primers: F-CACCTTCTGCAAAATTATGGCG, R-ACCTTTGCCAAGTCTAACTGTTAA); *ribosomal protein 18* (*rpS18*) (primers: F-GATTCCCGATTGGTTTTTGAATAG, R-AACCCCAA TAATGACGCAAACC); *TATA-box binding protein* (*tbp*) (primers: F-TGGCAGCAAGAAAGTATGCTAG, R-TCACATCACAGCTGCCTACC)) (Jeon et al., 2020; Mao et al., 2011; Moon et al., 2018; Mumoki et al., 2018). However, all of them showed a C_q value with SD higher than 1 (ZA-*rpS18* (SD = 3.49, *n* = 15), ZA-*cyp4g11* (SD = 2.59, *n* = 5), ZA-*eIF3-S8* (SD = 4.79, *n* = 6), ZA-*gapdh* (SD = 5.48, *n* = 3), ZA-*tbp* (SD = 5.35, *n* = 3)) and were considered unsuitable. Note that the qPCR reaction sample size between the genes varied substantially. This is as in the later course of the experiments, a gene was immediately excluded as reference gene if the SD was already considerable variable after three qPCR reactions, in order to save cDNA for the target gene qPCRs. Eventually, target gene expression in *A. mellifera scutellata* was quantified absolutely using an external calibration curve (Pfaffl, 2001).

In general, a strong influence of biotic and abiotic environment on gene expression has been reported across taxa (for review see Gibson, 2008). Along this line, the development of hypopharyngeal glands in honey bees is dependent on pollen type and the brood status of the colonies (Hrassnigg & Crailsheim, 1998; Huang et al., 1989; Omar et al., 2017; Standifer, 1967). In addition, differences in gene expression between subspecies have been shown for cultivated rice, *Drosophila willistoni* and the house mouse (Campbell et al., 2020; Ranz et al., 2023; Rottscheidt & Harr, 2007). However, environmental differences, for example season and plant species available, would certainly explain gene expression differences between European and African honey bees, but it does not explain why reference gene expression varies so much more in *A. mellifera scutellata* (ZA) compared to *A. mellifera* (DE).

But how can this stark difference in variation of "reference" gene expression be explained? When evaluating the data per age-group (Figure 2) it became obvious that while C_q values of freshly hatched South African bees in general did not differ significantly from C_q values of German bees (except for ZA-*pros26*-FH from DE-*pros26*-N, $p = .03$; ZA-*mrjp8*-FH from DE-*mrjp8*-F, $p = .03$ and ZA-*ppil2*-FH from DE-*ppil2*-N, $p = .02$), South African nurses and foragers had significantly higher C_q values, and thus a lower expression, than all other groups for all three genes analysed (except for ZA-*ppil2*-F from DE-*ppil2*-N, $p = .44$) (Figure 2) (one-way ANOVA; $df = 17, n = 66$, MS = 61.82, $F = 67.87$, $p < .0001$; with post-hoc Bonferroni tests, $p < .001$). Along this line, C_q values were not only affected by gene (*pros26*, *mrjp8* or *ppil2*) and sample origin (ZA or DE) but also by worker life stage (FH, N or F) (GLM; gene: MS = 187.36, $F = 205.68$, $df = 2$, $p < .0001$; sample origin: MS = 230.87, $F = 253.45$, $df = 1$, $p < .0001$; worker life stage: MS = 98.74, $F = 108.39$, $df = 2$, $p < .0001$). Thus, at some point after hatching *A. mellifera scutellata*-ZA workers significantly downregulated genes in their HPGs that were stably

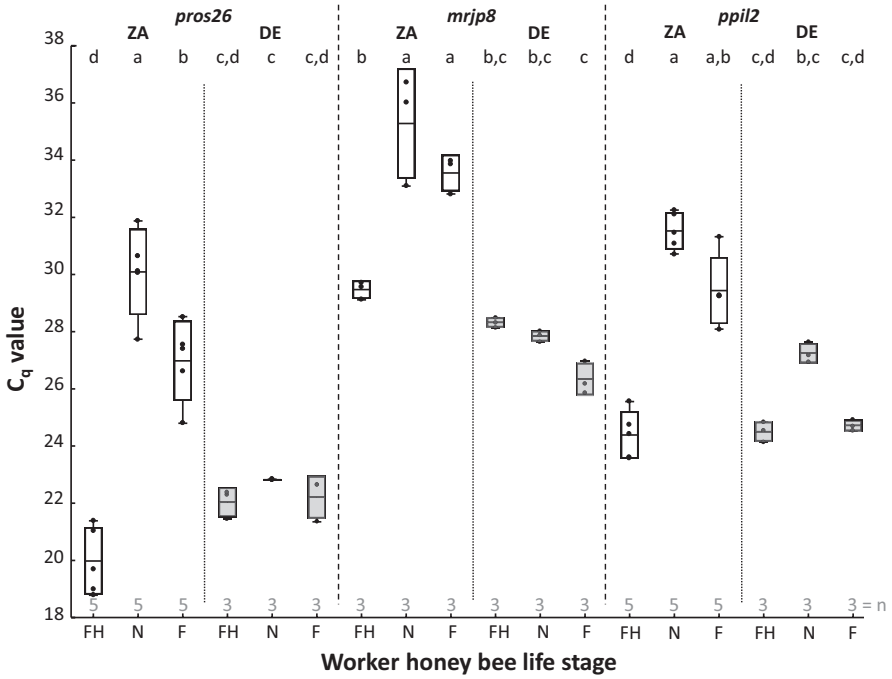

FIGURE 2 C_q values of validated genes according to worker honey bee life stage (FH, freshly hatched; N, nurse bee; F, forager bee). Boxes show mean ± standard deviation and whiskers show min-max. Individual data points are shown as circles. White boxes, ZA = *A. mellifera scutellata* sampled in South Africa; grey boxes, DE = *A. mellifera* sampled in Germany. Sample size (*n*) refers to pools of HPGs of 3 workers each. (a–d) C_q values of the same gene with different lowercase letters are significantly different (one-way ANOVA with post-hoc Bonferroni tests $p < .05$). C_q values for *mrjp8* are in ZA nurses and foragers so high (>32), that *mrjp8* would have been excluded as reference gene just for this reason alone.

expressed in *A. mellifera*-DE. This clearly points to another problem: The strong concentration of research on domesticated Western honey bees in the Northern hemisphere.

Even though *A. mellifera* is native to Europe, Africa and Western Asia (Ruttner, 1988), most studies refer to *A. mellifera* in Europe or to regions where it is not native. Of the 18,504 publications on *A. mellifera* indexed in the Web of Science Core Collection (WoS CC), 12,400 (67.0%) indicate at least one author from an area in which *A. mellifera* is native (Europe, Africa or Western Asia). Only 5.9% (1082) list at least one author with African affiliation (5.8% Western Asia, 55.4% Europe) (WoS CC search on 15.05.2023, search terms: "*Apis mellifera*" in all fields). Even if we generously assume that every publication that has at least one author with an African affiliation is about an African *A. mellifera* subspecies, this still represents an extremely large deficit in the number of publications in the main native distribution area of the Western honey bee (area Africa: 30.2 Mio. km^2, Europe: 10.5 Mio. km^2, Western Asia: 5.9 Mio. km^2) but reflects the situation in entomology, pollinator ecology and in research in general where studies undertaken in Africa make up just between 1%–4% of total articles published (Archer et al., 2014; Pandita & Singh, 2022; Sánchez-Bayo & Wyckhuys, 2019). Almost all of our knowledge on *A. mellifera* is on honey bees of the Northern hemisphere whereas "… the knowledge base of the honey bees of Africa is minute in comparison …" (Hepburn & Radloff, 1998). Thus, many of the certainly existing differences are not known, or there are hardly any studies on African honey bees. For example, queen larvae food jelly (royal jelly) of European honey bee breeds and subspecies is so well studied that based on those studies standards and standard methods exist to evaluate royal jelly quality (for review see Hu et al., 2019; Sabatini et al., 2009 and references therein) whereas only three studies focused so far on the composition of African honey bee royal jelly (El-Guendouz et al., 2020 (subspecies not given, Morocco); Maundu, 2004; Mokaya et al., 2020 (both *A. mellifera scutellata*, Kenya)). In addition, the dependence of hypopharyngeal gland development, as well as RNA and protein content, on age has been shown in a multitude of studies on European honey bees kept in the Northern hemisphere (for review see Ahmad et al., 2021; Crailsheim & Stolberg, 1989 [*A. mellifera carnica*, Austria]; Deseyn & Billen, 2005 [subspecies not given, Belgium]; Dobritzsch et al., 2019 [subspecies not given, Germany]; Knecht & Kaatz, 1990 [Carnolian hybrids, Germany]; Soudek, 1927 [*A. mellifera ligustica*, NY, USA]). In contrast to that, there are only few studies on African honey bee HPGs, which at least confirm the morphological results for three African subspecies (Al-Ghamdi et al., 2011 [*A. mellifera jemenitica*, Saudi Arabia]; Langlands et al., 2022 [*A. mellifera capensis* & *scutellata*, South Africa]). We can now substantiate these previously morphological based results with RNA amounts (Figure 3). As in workers sampled in Germany, RNA amount increased in HPGs of South African workers from freshly hatched to nurse bees ($p = .001$, $z = 3.94$) and decreased again in foragers ($p < .0001$, $z = 5.34$) (Figure 3) (Kruskal-Wallis ANOVA; $n = 72$, $H = 50.84$, $p < .0001$). Median RNA amounts were higher for ZA-nurses compared to DE-nurses (2.6-fold; DE 10.3 ± 3.9 µg, ZA 26.4 ± 8.7 µg) even though, due to the large IQR, this is not significant. It is unlikely that we missed the highest developmental point of HPGs in Dobritzsch et al. (2019) as workers were also collected on day 4 (RNA amount 9.0 ± 1.4 µg) and on day 12 (9.5 ± 1.6 µg) and day 8 had the highest median. The different regulation of the "reference" genes taken together with the higher RNA amount in nurse bees might indicate that development and secretion performance of HPGs differs between subspecies but there is not enough data on African honey bees to assert this.

One reason why statistical dispersion in our data is higher for ZA-workers compared to DE-workers (gene expression SD & RNA amount IQR) might be that beekeepers in Europe subjected

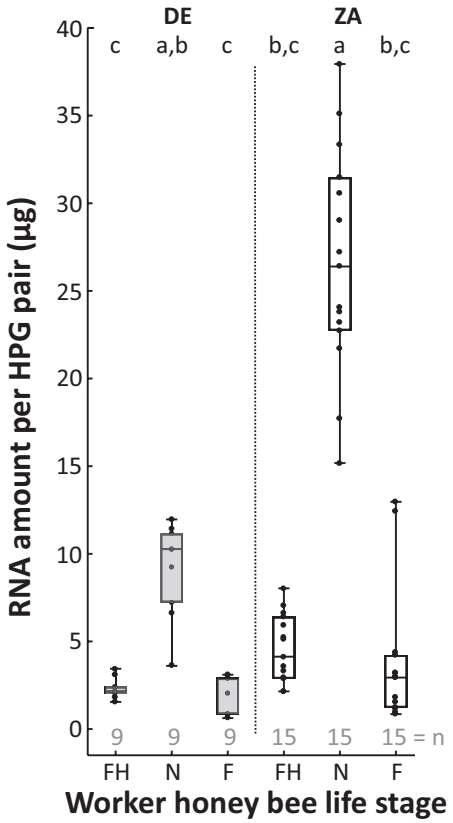

FIGURE 3 RNA amounts according to worker honey bee life stage (FH, freshly hatched; N, nurse bee; F, forager bee). Boxes show median ± interquartile range (IQR) and whiskers show min-max. Individual data points are shown as circles. White boxes, ZA = *A. mellifera scutellata* sampled in South Africa; grey boxes, DE = *A. mellifera* sampled in Germany. Sample size (*n*) refers numbers of individual workers. (a–c) RNA amounts with different lowercase letters are significantly different (Kruskal-Wallis ANOVA with post-hoc pairwise comparisons $p < .05$).

A. mellifera to selective breeding over decades, if not centuries whereas in Africa beekeeping still mostly involves trapping of wild swarms (Lowore et al., 2018; Requier et al., 2019). This is accompanied by a smaller genetic diversity in Europe than in Africa (Requier et al., 2019; Wallberg et al., 2014) which might explain the much more uniform gene expression. In addition, in Europe, wild *A. mellifera* populations are considered as extinct even though wild or feral colonies are still reported occasionally (Requier et al., 2019). In contrast to that, in Africa, more than 90% of the colonies are wild and managed honey bees constitute only a small proportion of the population (Pirk et al., 2016; Requier et al., 2019). However, in the Middle-East extensive introgression of the European subspecies *A. mellifera caucasica* is detected, likely caused by selective breeding as well (Alburaki et al., 2013). Thus, selective breeding of European *A. mellifera* and their domestication might have led to a decrease of gene expression diversity as it did in other domestic species compared to their wild progenitors such as dog, silkworm, chicken, rice, common bean and cotton (Bellucci et al., 2014; Liu et al., 2019). Domestication is in general accompanied by drastic phenotypical and physiological changes for many species (Darwin, 1868), so why should the Western honey bee be an exception?

In addition to the difficulty of finding reference genes, there is another issue that poses problems when analysing qPCR data—very different amounts of RNA in the samples as shown in Figure 3. The initial motivation that ultimately led to the development of qPCR came originally from clinical diagnostics in the 1990s (Higuchi et al., 1992) and nowadays qPCR is widely used in clinical microbiology (Kralik & Ricchi, 2017). Clinical samples for qPCR include often specific amounts of whole blood or swab material (Ma et al., 2021), for cell culture studies cells are seeded in specific densities into wells (Van Peer et al., 2012) and from tissue samples of bigger species RNA is usually isolated from a specific amount of tissue. Thus, presumably, gene expression is often, even though certainly not always, compared between samples from which roughly the same amount of RNA can be isolated. However, when it comes to smaller specimen, for example insects, gene expression is regularly compared between sterile castes and reproductives, and between body parts or organs often of different size (Buttstedt et al., 2013; Dobritzsch et al., 2019; Dong et al., 2023; Guidugli-Lazzarini et al., 2008; Mao et al., 2015). This results certainly in vastly different amounts of total RNA isolated, as from the HPGs (Figure 3), which is seldom reported. Whereas it is meanwhile common knowledge and specified in a variety of qPCR guidelines that RNA quality and an equal amount of RNA for cDNA synthesis is crucially important, differences in isolated RNA amount from the starting material are neglected (Bustin et al., 2009; Fleige & Pfaffl, 2006). However, this is crucially important to consider as a higher amount of total RNA in for example the same organ at a specific life stage can drastically dilute the difference in expression of a specific gene between the two life stages and it also interferes with reference gene validation.

Let us assume that a given organ contains five times the amount of RNA at life stage II compared to life stage I (Figure 4). Then the difference in abundance of specific (target) mRNAs will be 5-fold diluted in cDNA synthesised from the same amount of RNA. Potential reference genes that have actually the same amount of mRNA molecules at both life stages in the organ (blue in Figure 4) will produce different C_q values in qPCR and be sorted out as non-suitable. In contrast, reference genes that have a 5-times higher number of molecules at the life stage with the 5-times higher amount of RNA (green in Figure 4) will result in equal C_q values and thus chosen as reference genes. This will eventually result in a 5-fold underestimation in the difference of relative target gene expression between the two life stages (pink in Figure 4) and likely also result in false negatives. Thus, when gene expression is compared between organs/body parts that have different amounts of RNA and the primary goal is to eventually compare how much more mRNA of a specific gene is produced in the whole organ/body part, then it has to be corrected for RNA amount. This can be done in two ways: Either reference genes should be chosen that directly reflect the difference in RNA amount, like the blue one in Figure 4, or reference genes with stable C_q values should be chosen which are basically "upregulated" along with the total RNA but then relative gene

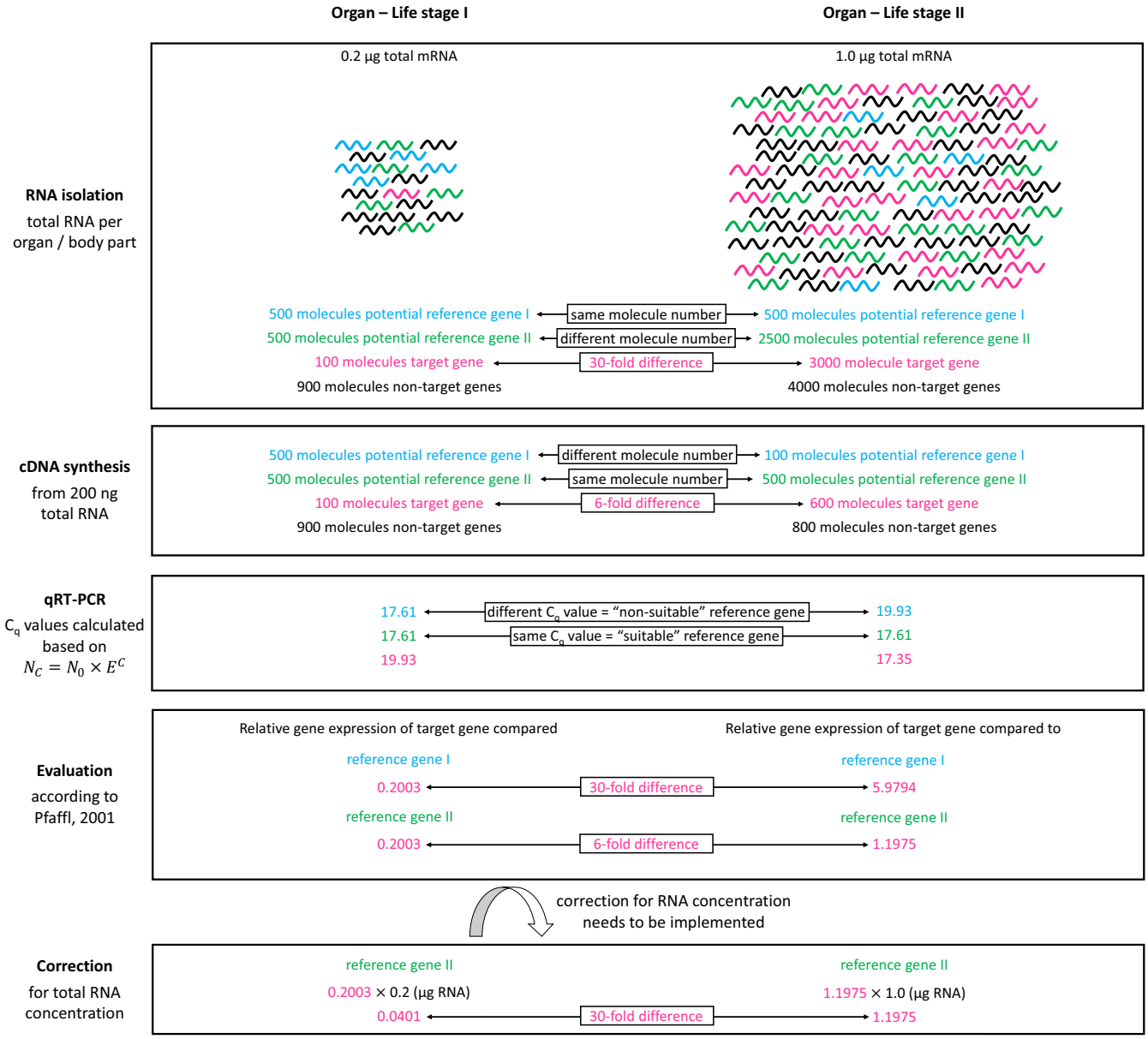

FIGURE 4 Influence of different amounts of total RNA on evaluation of gene expression. PCR efficiency was assumed to be 2.0 for the calculations.

expression values have to be multiplied with RNA amounts as done in Dobritzsch et al. (2019). Of course, there is a caveat of using total RNA mass for correction, which is the fact that total RNA is not always representative of the mRNA fraction as it contains predominantly rRNA (Vandesompele et al., 2002). This is the reason why using total RNA amount alone for normalization has been excluded in the first place in the favour of internal control genes (Vandesompele et al., 2002). However, we do not suggest to only use total RNA for normalization but normalise first in relation to a reference gene and then correct for RNA amount.

One reason that might influence the higher C_q values in ZA-nurse bees compared to ZA-freshly hatched bees is the higher RNA amount in nurse bees, which might simply "dilute" the reference genes. However, a C_q value difference of about 10 (for *pros26*) is much higher than one would expect for a 2.6-fold difference in RNA amount. Furthermore, it does not explain why C_q values are also much higher in ZA-foragers compared to ZA-freshly hatched bees even though here RNA amount does not differ (Figure 3) and it does certainly not explain why this effect is not seen in DE-workers.

Thus, we are back at the question of how to find reliable reference genes in the first place. For example, geometric averaging of multiple internal control genes was beneficial for accurate normalization of gene expression earlier (Vandesompele et al., 2002). However, this requires that the potential reference genes are not coregulated which is certainly the case for our data. Another technique that has been successfully used to predict reference genes in a variety of species is full transcriptome sequencing (Alexander et al., 2012; Vallier et al., 2023;

Zhang et al., 2020). Instead of searching for differentially expressed genes, available transcriptomes are screened for genes that are never regulated across studies. This is certainly also a possibility in the model organism *A. mellifera* as a search in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) for "Apis mellifera transcriptome" resulted in 2415 hits (search on 25 July, 2023; https://www.ncbi.nlm.nih.gov/sra/?term=apis+mellifera+transcriptome) and thus sufficient amount of transcriptome data should be available. However, only 12 out of these 2415 were performed on African honey bee subspecies [*A. mellifera capensis*, *A. mellifera scutellata*, BioProject PRJNA438231 (Aumer et al., 2018)]. Thus, even though using full transcriptome data on non-African *A.mellifera* is likely better for reference gene selection than using arbitrarily selected reference genes published for non-African *A. mellifera*, it is still quite suboptimal when virtually no data are included in transcriptome-based reference gene selection of the subspecies for which the reference genes are intended. Still, choosing and revalidating reference genes from full transcriptome data might likely result faster in suitable reference genes. However, in case suitable reference genes cannot be found, and especially from small samples, RNA and thus cDNA amount might be rather limited to test through a variety of reference genes, gene expression should be quantified absolute based on standard curves amplyfying known amounts of target DNA (Bustin, 2000; Rutledge & Côté, 2003).

Thus, we recommend the following: When working with species that have a very wide distribution, like *A. mellifera*, the subspecies should always be mentioned. If the subspecies is unknown, or the individuals in questions are unknown hybrids, this should be stated openly. Furthermore, the sample location should be given in addition. This is of particular importance for very widely distributed subspecies, which includes most of the African subspecies, as gene expression might already be different between local populations. Furthermore, the RNA concentration should be mentioned and in case the goal is to eventually compare transcript amount between total organs, the RNA concentration should be corrected for. And irrespective of whether or not a gene has been published as reference gene for the given species, as soon as something changes, for example subspecies, age of the individuals, treatment, or season, the reference gene should be re-validated for suitability. In fact, reference genes should simply be re-validated in any case. When they are anyways already measured, it is certainly no further issue to check for standard deviations. If no suitable reference genes can be found, which becomes more likely the more different groups are included in the analysis, gene expression should be evaluated absolute.

AUTHOR CONTRIBUTIONS

All authors conceived and designed the study and contributed to reviewing the final manuscript. AB collected the data, AB and AAY analysed the data, AB wrote the first version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY AND BENEFIT-SHARING STATEMENT

Data values (C_q & RNA amounts) are shared in Figures 1–3.

ORCID

Anja Buttstedt https://orcid.org/0000-0003-2299-7000

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