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Identification of the *Rdl* mutation in laboratory and field strains of the cat flea, *Ctenocephalides felis* (Siphonaptera: Pulicidae)

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Abstract: In many insect species, resistance to cyclodiene insecticides is caused by amino acid substitutions at a single residue (A302) within the M2 transmembrane region of the γ-aminobutyric acid (GABA) receptor sub-unit termed Rdl (resistance to dieldrin). These mutations (A302S and A302G) have also been shown to confer varying levels of cross-resistance to fipronil, a phenylpyrazole insecticide with a similar mode of action to cyclodienes. To investigate the possible occurrence of these mutations in the cat flea, *Ctenocephalides felis* (Bouché), a 176-bp fragment of the cat flea *Rdl* gene, encompassing the mutation site, was PCR amplified and sequenced from nine laboratory flea strains. The A302S mutation was found in eight of the nine strains analysed, although the relative frequency of the mutant allele varied between strains. Only one strain (R6) was found to be homozygous for the S302 allele in all the individuals tested, and this correlated with previous reports of low-level fipronil resistance in this strain. A PCR-based diagnostic assay, capable of screening individual fleas for this mutation, was developed and used to survey a range of fleas collected at random from veterinary clinics in the UK and USA. The A302S mutation was present at a high frequency in these domestic pet populations.

Keywords: cat flea; *Ctenocephalides felis*; insecticide resistance; Rdl; cyclodiene; fipronil

1 INTRODUCTION

The cat flea, *Ctenocephalides felis* (Bouché) is the most important ectoparasite of domestic cats and dogs world-wide. It is responsible for bite allergy dermatitis and is known to transmit the dog tapeworm.[1] The normal method of controlling fleas is through the application of chemical insecticides, and the widespread use of these over many years has led to reports of resistance to 'older' chemical classes such as organophosphates (OPs), carbamates, pyrethroids and cyclodienes.[2–5] Although increased metabolism and target site insensitivity have been implicated in causing this resistance,[6] little information is available regarding the specific molecular mechanisms involved.

Previous work on agricultural pests and human disease vectors has shown that insecticide resistance often develops as a result of structural changes in the target protein that render it less sensitive to the effects of the insecticide. An example of this is resistance to cyclodienes, which arises by substitutions at a single residue (Alanine302) in the M2 region of a γ-aminobutyric acid (GABA)-gated chloride channel, encoded by the *Rdl* (*resistance to dieldrin*) gene.[7] Two resistant *Rdl* alleles (A302S and A302G) have now been identified in a wide range of insect species including the fruit flies *Drosophila melanogaster* Meigen and *Drosophila simulans* (Sturtevant), housefly, *Musca domestica* L, American cockroach, *Periplaneta americana* (L), red flour beetle, *Tribolium castaneum* (Hbst), the mosquitoes *Aedes aegypti* L and *Anopheles stephensi* Liston, coffee berry borer, *Hypothenemus hampei* Ferrari, German cockroach, *Blattella germanica* (L), whitefly, *Bemisia tabaci* Gennadius and peach potato aphid, *Myzus persicae* (Sulzer) (see Reference 8 and references therein). Although cyclodienes have been largely withdrawn from use as insecticides, the presence of this mutation may continue to be selected because it confers varying levels of cross-resistance to fipronil, a phenylpyrazole insecticide which also acts on the GABA-gated chloride channel.[9,10]

To investigate recent reports of reduced fipronil efficacy for a cat flea strain collected in Florida,[11,12]

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we have cloned and sequenced the exon 7 region of the *Rdl* gene (encompassing M2) from a range of laboratory flea strains. The resistance mutation, A302S, was present at varying frequency in all except one of these strains, including the strain from Florida in which all individuals were homozygous for the resistant allele. We also describe the development of a PCR-based diagnostic assay that can rapidly type single fleas for the presence/absence of the mutation, and the application of this technique to survey fleas collected from veterinary clinics in the UK and USA.

2 EXPERIMENTAL

2.1 Cat flea strains

A total of nine laboratory strains was used in this study. Four strains originating from the USA (Cruthers, UCR, Auburn and Cottontail) and one from Germany (Monheim) were reared on cats at Bayer Animal Health; a UK strain (RVC) was provided by Professor D Jacobs (Royal Veterinary College, London); the fipronil-resistant strain, R6[11] was provided by Novartis Animal Health and two further USA strains (ARC and KS1[12]) were provided by Dr M Dryden (Kansas State University). Domestic pet flea strains (referred to in this text as 'field collections') were collected from veterinary clinics in the USA and UK through the Advantage Monitoring Program[13] and provided by M Hutchinson and C Booler (Royal Veterinary College, London).

2.2 Cloning and sequencing of *Rdl* gene fragments

Genomic DNA was extracted from 20–30 adult cat fleas using the Phytopure DNA Extraction Kit (Nucleon Biosciences Ltd) according to the manufacturer's instructions. To PCR amplify exon 7 (encoding transmembrane region M1/M2) of the cat flea *Rdl* gene, degenerate oligonucleotide primers were designed against conserved peptide motifs within the sequences of other known insect Rdl sub-units (Rdl1-3 and G1, Table 1). A primary PCR was performed using primers Rdl1 and Rdl2, followed by secondary, nested PCRs using primers Rdl1 and Rdl3 or G1 and Rdl2. This two-step PCR protocol greatly increased the selectivity of the reaction and amplified single fragments of the expected size (142 bp or 176 bp, see Fig 1A). All PCR reactions contained 0.2 mM dNTPS, 100 ng of each primer, 1.5 units of *Taq* polymerase and 50–100 ng of genomic DNA, and were run for 30 cycles with 30 s at 94 °C, 50 s at 50 °C and 90 s at 72 °C. PCR fragments were initially recovered from agarose gels and cloned using the pGEM-T Easy Vector System (Promega) for sequencing. Once the sequence had been established for each strain, a 3′ flea Rdl-specific primer (Rdl4, Table 1) was used for direct sequencing of PCR products through the mutation site. All sequencing reactions were performed using ABI PRISM BigDye Terminator Sequencing kits and analysed on a 310 Automated DNA Sequencer (PE Applied Biosystems).

Table 1. Primer sequences used in this study[a]

Primer name	Sequence
Rdl1	WSN TGG GTN WSN TTY TGG
Rdl2	GCR AAN ACC ATN ACR AAR CA
Rdl3	CRT CDA TNS WYT TNA CRT A
Rdl4	TAG ATT TGA CGT ATG ATA TT
G1	TSK TGG GTW WST TTT TGG CTG
ANSF (allele non-specific forward)	GGT TTC TTT TTG GCT GAA TCG
ANSR (allele non-specific reverse)	CAG GTG CCC AGG TAG ACG TC
RASR (resistant allele-specific reverse)	TGT CAC CAG TGA GGC TCT CT
SASF (susceptible allele-specific forward)	ATG CTA CAC CAG CTC GAG TCG

[a] All primers are shown 5′ to 3′. Degenerate bases are represented using standard IUB codes: R = A + G, Y = C + T, D = A + G + T, W = A + T, S = G + C, M = A + C, N = A + C + G + T.

2.3 Bi-directional PCR amplification of specific alleles (bi-PASA)

The bi-PASA protocol for detecting the cat flea *Rdl* alleles was based on that reported previously.[14–16] Genomic DNA was extracted from single fleas using DNAzol genomic DNA isolation reagent (Molecular Research Center, Inc) at one-fifth of the recommended rate. Four primers were used in each reaction, two outer primers (ANSF and ANSR) that are not allele selective, and two inner primers (SASF and RASR) that are selective for the A302 and S302 alleles, respectively, at their 3′ bases (see Table 1 and Fig 2). The ANSF and ANSR primers amplify a control fragment of 155 bp from both *Rdl* alleles, whereas the 3′ selectivity of the SASF and RASR primers means that they will only amplify fragments if their matching 3′ base (and hence allele) is present. These primers point in opposite directions and give different size fragments of 65 bp (SASF, A302 allele) and 130 bp (RASR, S302 allele) in combination with the ANSF and ANSR primers (see Fig 2), so that both alleles can be scored in a single PCR reaction. To enrich the template prior to the bi-PASA reaction, a primary PCR was carried out using primers Rdl2 and G1 according to Section 2.2. The bi-PASA reactions contained 1 µl of the primary reaction together with 150 ng of primers ANSF and RASR, 100 ng of primers ANSR and SASF, 0.2 mM dNTPs, 1.5 mM magnesium chloride and 1.5 units of *Taq* polymerase. PCR was performed for 30 cycles of 30 s at 94 °C, 50 s at 55 °C and 90 s at 72 °C. Bi-PASA products were separated on 20% pre-cast polyacrylamide TBE gels (Invitrogen) and visualised by ethidium bromide staining. The bi-PASA protocol was optimised, and its accuracy confirmed, by running gDNA templates that had been previously genotyped by DNA sequencing. These samples were then used as controls for the analysis of field collections.

3 RESULTS

The degenerate *Rdl* gene primers successfully amplified a 176-bp fragment from cat flea genomic DNA. Cloning and sequencing confirmed that this was indeed the exon 7 (M2) region of the flea Rdl-type GABA receptor sub-unit since the sequences of known insect Rdl sub-units are very highly conserved across this region (Fig 1, EMBL accession AJ 717584). A single point mutation (GCT to TCT) causing the same alanine to serine mutation, A302S, previously shown to be associated with cyclodiene resistance in other insects, was found in most of the laboratory cat flea strains analysed (Fig 1A, mutation numbering according to the *D melanogaster* Rdl sequence[7]). This was the only base difference observed in this region following repetitive sequencing of multiple individuals from each strain. The A302/S302 genotypes of 20–22 individuals of each strain, as determined by direct DNA sequencing of PCR products from single fleas, are shown in Table 2. The relative frequency of the S302 (resistant) allele varies from strain to strain with the highest frequency occurring in the R6, Cruthers, Monheim and Cottontail strains, with the R6 strain homozygous for the mutant allele in all individuals tested. ARC and Auburn showed the lowest frequencies of this allele, with ARC being homozygous for the A302 (susceptible) allele. The remaining strains (RVC, UCR and KS1) were mixed populations with most individuals scored as heterozygotes.

The widespread occurrence of the *Rdl* mutation within the laboratory strains suggested that this mutation/allele arose several years ago and would therefore be likely to be common in domestic pet populations (see Section 4). This was tested by

Table 2. Rdl genotypes of laboratory flea strains

		Genotype[a]		
Strain	n[b]	S/S	S/R	R/R
Cottontail	22	5	6	11
Auburn	20	15	3	2
Monheim	20	2	6	12
Cruthers	20	0	5	15
RVC	20	0	15	5
UCR	20	0	18	2
KS1	20	0	13	7
ARC	20	20	0	0
R6	20	0	0	20

[a] S = A302 allele. R = S302 allele.
[b] Number of individuals tested.

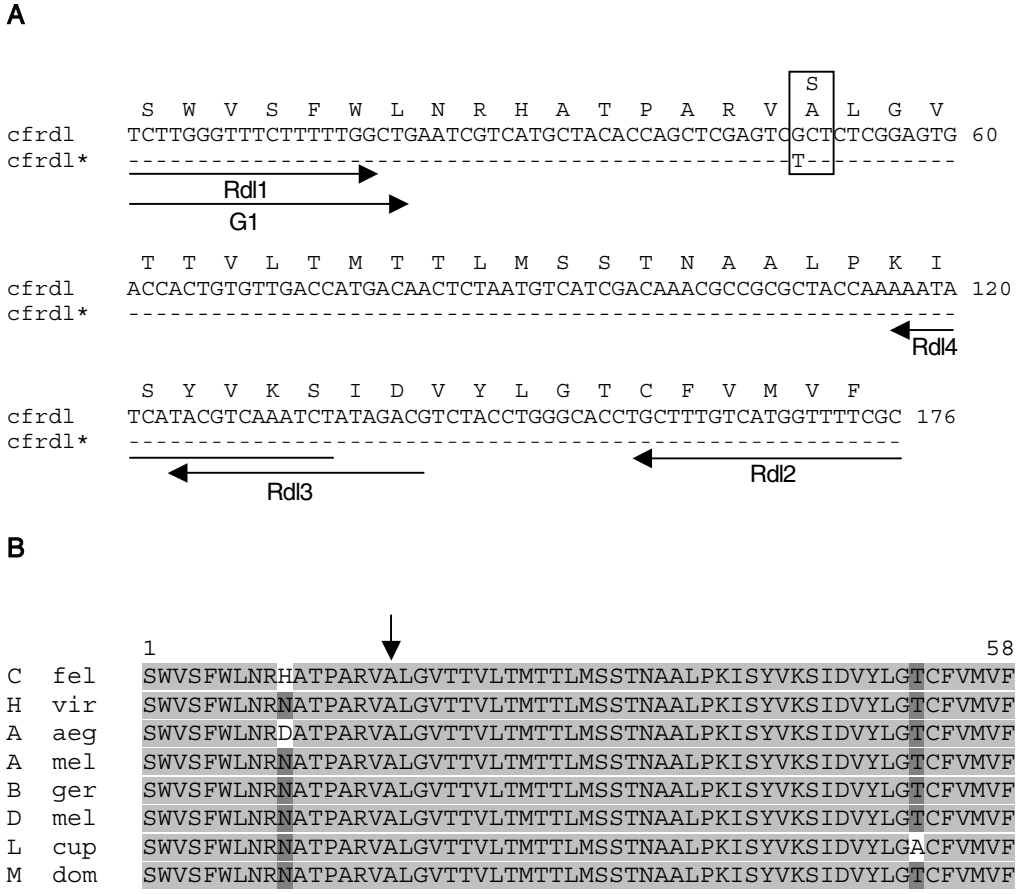

Figure 1. A. Nucleotide and amino acid sequence of the exon 7 region of the cat flea *Rdl* gene. cfrdl and cfrdl* represent the A302 and S302 alleles. The position of the A302S mutation is boxed. The locations of primers used in PCR amplification and DNA sequencing are indicated by arrows. **B**. Alignment of the cat flea Rdl sequence with the corresponding region from other insect species. The position of A302 is indicated by the arrow. Additional sequences were taken from the UniProt database: *Heliothis virescens* (O18471), *Aedes aegypti* (Q16896), *Apis mellifera* (077249), *Blattella germanica* (Q26411), *Drosophila melanogaster* (P92138), *Lucilia cuprina* (O17145) and *Musca domestica* (076472).

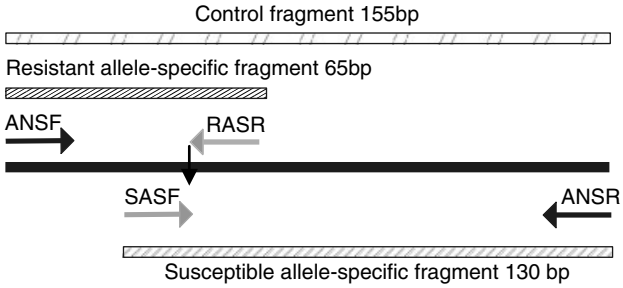

Figure 2. Schematic diagram of the Bi-PASA reaction. The four primers indicated by arrows are RASR: resistant allele-specific reverse primer, SASF: susceptible allele-specific forward primer, ANSF: allele non-specific forward primer and ANSR: allele non-specific reverse primer. See text for details.

screening fleas collected from domestic pets using an allele-specific PCR assay (bi-PASA) that was designed to give rapid unambiguous scoring of individual A302/S302 genotypes. Because the allele-selective primers used in this assay anneal to opposite strands of the gene and amplify in opposite directions, they can be used to generate differently sized fragments, thereby allowing both alleles to be scored in a single reaction (Fig 2; note that standard PASA methods require two separate reactions to score each allele[17]). As shown in Fig 3, for 'control' samples of known genotype, the homozygous susceptible and resistant individuals are distinguished by the presence of either the larger 130-bp, or smaller 60-bp fragment, respectively. The heterozygote has both fragments and all genotypes have the allele non-specific 'control fragment' of 155 bp. The technique was applied to individual fleas from several domestic pet strains collected from veterinary clinics in the USA and UK. In a preliminary survey, 30 fleas collected from three clinics in the USA (10 fleas from each clinic) and 113 fleas from 14 clinics in the UK (3–4 from each clinic) were tested. The assay gave unambiguous scoring of both alleles in all of the individuals tested (see examples in Fig 3), with the mutant S302 allele present at high frequency in both USA and UK pet populations (Table 3). Although the overall frequency of the S302 allele was 68% in the UK sample (55 homozygotes and 45 heterozygotes from 113 tested) and 55% in the USA sample (9 homozygotes and 15 heterozygotes from 30 tested), the lack of treatment history for the pets involved means that it is difficult to conclude at this stage whether this is a direct consequence of the recent widespread use of fipronil for flea control on domestic pets (see Section 4).

Table 3. Rdl genotypes of field-collected fleas

		Genotype[a]		
Country	n[b]	S/S	S/R	R/R
UK	113	13	45	55
USA	30	6	15	9

[a] S = A302 allele. R = S302 allele.
[b] Number of individuals tested.

4 DISCUSSION

We have identified two distinct *Rdl* alleles associated with the exon 7 region of the cat flea *Rdl* GABA receptor sub-unit gene: the wild-type allele containing alanine302 and a second allele in which the alanine is replaced by serine (A302S), a mutation previously shown to confer resistance to cyclodiene insecticides in a range of insect species.[8] The relative frequencies of these alleles varied among the nine laboratory strains tested, with two strains, ARC and R6, homozygous for the wild-type (A302) and mutant (S302) alleles respectively, and the others containing mixed populations of the two alleles.

The discovery of the mutant S302 allele within so many of the laboratory strains was unexpected, since most of the strains have been held in laboratory culture for several years and have not undergone routine treatment (and therefore selection) with cyclodienes or other insecticides. Furthermore, the cyclodienes have been largely withdrawn from use as commercial insecticides, and we are not aware that they were ever used widely for the direct control of fleas. Nevertheless, these compounds have been used in various home (eg cockroach, ant and termite) and garden applications, and this, combined with their

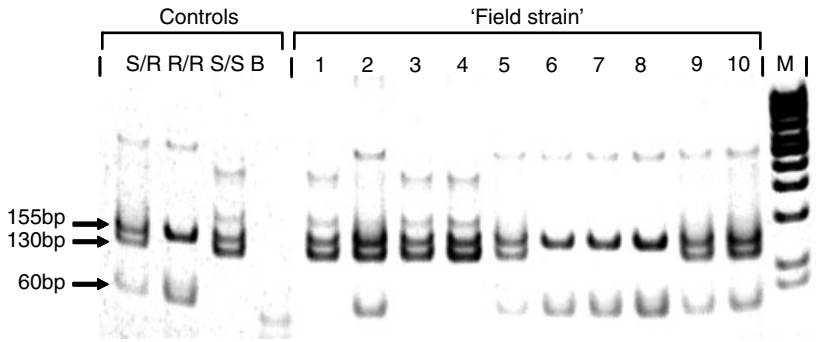

Figure 3. Bi-PASA reaction products for *Rdl* genotyping of individual cat fleas. The 155 bp 'control' fragment is amplified in all; the 130 bp and 60 bp fragments are selectively amplified when A302 (S) and S302 (R) alleles are present. Standards of known genotype are shown on the left, the remainder (1–10) are field-collected fleas. Lane B (blank) is a no template DNA control, lane M is a standard size marker in which the lower 3 bands are 80 bp, 100 bp and 200 bp.

environmental persistence, makes it very likely that these fleas would have undergone significant, indirect exposure to cyclodienes in the past, prior to their establishment as laboratory strains. The persistence of the mutation in laboratory culture, without selection, suggests that it either has a low fitness cost in cat fleas, or that any such cost is not realised in the laboratory environment. This is consistent with reports in other insects where an apparent absence of fitness costs has led to persistence of the *Rdl* mutation in both laboratory and field strains.[18,19]

The presence and apparent stability of the mutant allele within the laboratory flea strains raises interesting questions in relation to its frequency within current domestic pet flea populations, and the effect that it might have on the efficacy of newer control products, such as fipronil, that have a similar mode of action at the insect GABA-gated chloride channel.[20] Previous studies in other insects have shown that resistance to cyclodienes is associated with a low level of cross-resistance to fipronil in houseflies,[9,10] mosquitoes[21] and German cockroaches.[10,19] Furthermore, functional expression studies with mutant (A302S) Rdl GABA receptors show a marked reduction in affinity to fipronil when compared to wild-type receptors.[22] In this context, our analysis of the three strains used in the study by Payne *et al*[12] (R6, KS1 and ARC) is especially interesting since here we are able to compare information on the A302/S302 genotypes of these strains with *in vivo* bioassay data demonstrating their relative levels of susceptibility to fipronil. Payne *et al* showed that the R6 strain (which we find to be homozygous for the mutant S302 allele) was less susceptible to fipronil on treated cats than the other two strains, with over 20% adult flea survival for the R6 strain at 30 days post-treatment. The ARC strain (homozygous for the wild-type A302 allele) was the most susceptible to fipronil, with no adult flea survival 30 days post-treatment; whilst the KS1 strain (heterogeneous for the A302/S302 alleles with most individuals heterozygous for the mutation) showed an intermediate susceptibility with 10% survival at 30 days post-treatment. In a separate study, Schenker *et al*[11] also showed the R6 strain to be significantly less susceptible to fipronil treatments than a control susceptible strain, with over 50% and 80% of adult fleas surviving fipronil spot-on or spray treatments at 30 days. These authors also showed the R6 strain to be 25-fold more resistant than the laboratory susceptible strain using an *in vitro* filter strip test. Taken together, these results clearly add weight to the hypothesis that cyclodiene resistance and the S302 mutation do indeed confer some degree of cross-resistance to fipronil in fleas. However, it should be noted that the clinical consequences of this 'resistance' in terms of flea control by fipronil in domestic pet populations is less clear at present. In both of the above studies, although reduced control was seen on cats at 28/30 days post-treatment, highly effective control was nevertheless seen at shorter periods after treatment (up to 20 days), where almost 100% control was achieved even for the R6 strain.[11,12]

Given the unexpectedly high occurrence of the S302 mutation in the laboratory strains and the recent widespread use of fipronil to control cat fleas, we were keen to investigate the incidence of the S302 mutation within current domestic pet flea populations. To facilitate this, we have developed a PCR-based diagnostic assay (termed bi-PASA) that can be used to accurately genotype large numbers of individual adult fleas for the presence/absence of the mutation. The assay uses allele-specific primers that are complementary to either the wild-type (alanine, *G*CT) or mutant (serine, *T*CT) codon at residue 302 of the *Rdl* gene, and are designed to amplify differently sized fragments in a single reaction that can then be separated and scored by gel electrophoresis.

In a preliminary survey of current domestic pet populations, we used the assay to genotype fleas collected at random from 14 veterinary clinics in the UK and a further three clinics in the USA. This survey showed that the frequency of the mutant S302 allele is indeed high in both populations tested with almost 50% of the UK fleas scored as homozygous for this allele (Table 3). Although the frequency was unexpectedly high, the random nature of these collections and lack of detailed treatment histories for the pets involved makes it difficult to draw definite conclusions as to the reason(s) for this. As mentioned above, possible factors might include (1) a lack of fitness cost associated with this allele, resulting in it being retained at high frequency in the wake of past, indirect exposure to cyclodienes; (2) recurrent, indirect exposure to these compounds as a result of their environmental stability and ongoing occasional use around the home; or (3) direct, albeit weak, selection with fipronil which is now one of the main products used for controlling fleas on domestic pets. At this stage, it is not possible to distinguish between these possibilities, and it is difficult, therefore, to predict whether the high frequency of the mutation in flea populations is likely to have any major clinical consequences for the long-term, effective use of fipronil in flea control. Clearly, more detailed investigations are needed to establish or reject any link between the incidence of the mutation and observed treatment failures with fipronil in domestic pet populations, and, in this respect, the bi-PASA assay should provide a reliable and robust tool for quickly and unequivocally genotyping large numbers of fleas.

Finally, as the costs of developing and running DNA-based diagnostics continue to fall, and their reliability and suitability for automation improve, it becomes increasingly likely that such tools will become an integral and routine part of resistance detection and management in the future. This is particularly true for target-site mechanisms such as *Rdl*, where resistance is caused by point mutations in large and complex membrane proteins for which there are no simple

biochemical assays for assessing target sensitivity. In insects where resistance is well characterised and multiple mechanisms are known to occur, these DNA-based mutation detection assays can be run alongside existing biochemical assays for metabolic resistance to quickly establish a 'resistance profile' for individual insects that then enables a more informed choice of those compounds that are most likely to retain good activity based on the mechanism(s) present.[23]

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NOTE ADDED IN PROOF

Related results have recently been reported by Daborn *et al*.[24]

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